Tissue distribution and function of arginase I : an experimental study

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ROLE OF ARGININE IN MACROPHAGE FUNCTION

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Abbreviations

Standard nomenclature is used to distinguish between protein and RNA expression, *e.g*. *Arg1* denotes the murine arginase 1 mRNA or gene, *ARG1* the human mRNA or gene, and ARG1 both the human and murine protein. When referring to both mRNA and protein simultaneously, the full name arginase 1 is used.

ARG1	arginase 1
CAT2B	cationic amino acid transporter 2b a.k.a. solute carrier family 7a2b (SLC7A2B)
CHI3L3	chitinase3-like protein3, a.k.a YM1
DFMO	difluoromethylornithine
GCN2	general control non-derepressible kinase 2, a.k.a eukaryotic translation initiation factor 2-alpha kinase 4, EIF2AK4
IFNγ	interferon gamma
IL	interleukin
IL4R	interleukin receptor
MRC1	mannose receptor, C type 1
NOS2	nitric oxide synthase 2, a.k.a inducible nitric oxide synthase
ODC	ornithine decarboxylase
PBMC	peripheral blood mononuclear cells
PEG	polyethylene glycol
RETNLB	resistin-like β, a.k.a FIZZ1
SSAT	Spermidine/spermine N^1-acetyltransferase
SMO	spermine oxidase
TNFα	tumor necrosis factor alpha
VSMC	vascular smooth muscle cells

Keywords

arginine metabolism, alternative activation, polyamines, arginine transport, inflammatory disease

Keypoints

- Macrophage classification *in vivo* should be based on function
- Arginine metabolism and transport in macrophages serves to control ambient arginine
- Control of local arginine availability is a mechanism to limit inflammation and fibrosis
- Macrophage arginase 1 does not play a role in polyamine synthesis
- Macrophage arginase 1 does not play a significant role for proline synthesis to support collagen deposition

Role of arginine in macrophage function

Macrophages

Macrophages are gluttons with daily garbage clearance and recycling among their major functions in the body. They phagocytose up to $2x10^{11}$ erythrocytes per day in the spleen, preserving the iron from hemoglobin for reuse, and they remove cellular debris and apoptotic cells everywhere else. The enormous importance of this task is reflected by the huge numbers of macrophages (10-15% of all cells) that are found in almost every tissue (1). If one assumes the recently published estimate of $3,72x10^{13}$ cells in a human body (2), this amounts to ~$5x10^{12}$ macrophages. Although the phagocytic function clearly was the inspiration for their name, macrophages are usually associated with their role in innate and adaptive immune defense. Accordingly, a Pubmed inquiry with the search terms "macrophages" and "immunity" results in ~37,800 hits, whereas "macrophages AND phagocytosis NOT immunity" delivers only ~3460 references (August 2014).

Macrophages are a heterogeneous cell population and are classified according to their tissue distribution, their activation status and their expressed surface markers, depending on the interests of the researcher or the methods applied. None of these criteria is permanent, since tissue distribution, activation status and surface markers can change rapidly in response to environmental triggers, such as cytokines, infectious agents, and foreign substances. Nevertheless, the activation status is the most relevant characteristic for the function of macrophages in innate and acquired immunity. In accordance with the T_H1/T_H2 nomenclature, macrophages have been classified as M1 and M2, or classically and alternatively activated macrophages, respectively (3). M1 macrophages show enhanced microbiocidal and tumoricidal capacity, and are induced by IFNγ and LPS or TNFα, whereas M2 macrophages are often activated by IL4 and IL13 and support T_H2-associated effector functions, play a role in wound healing, and in parasite killing. The M2 label has been further subdivided into M2a, M2b and M2c subpopulations (4,5) based on the stimuli that are required for their activation:

- M2a: IL4/IL13-elicited alternatively activated macrophages;
- M2b: induced by immune complexes, Toll-like receptor (TLR) agonists or IL1R agonists; and
- M2c: activated by IL10 and glucocorticoid hormones.

Macrophage subsets 2a-2c share some common functional properties, such as low IL12 expression and involvement in T_H2 responses and are, therefore, often collectively named M2 macrophages. According to some references, all M2 subpopulations show increased IL10 expression (5,6), but others assign high IL10 expression only to subpopulations 2b and 2c (7,8). The evolving view is that M1 and M2 polarization are the extremes of a continuum, that macrophages can switch activation states, and intermediate forms exist (3,8,9). Pure M1- or M2-polarized macrophages probably only exist in culture where well-defined triggers are used but not *in vivo* where the cells are exposed to a multitude of triggers in combinations and concentrations which differ in

every tissue and with every type of infection. In addition, the often described plasticity of macrophages allows them to change from one activation status to another provided the appropriate triggers are delivered by the environment as has been demonstrated *in vitro* (10,11) and *in vivo* (12-14), and reviewed extensively (15,16).
In addition to their highly desirable protective immunological and repair functions, all macrophage populations named above also aggravate pathological situations by supporting excessive inflammation (M1), or tumor growth, tissue fibrosis and allergy (M2).

Since macrophages grouped under M2 include cells with remarkable differences with respect to gene expression and physiologic function on the one hand, and since macrophages with similar expression profiles of marker genes can be elicited by quite diverse stimuli on the other hand (see below), a classification based on fundamental functions has also been suggested (8). This classification distinguishes:

- macrophages involved in host defense, which are identical to the classically activated or M1 macrophages,
- wound-healing macrophages that are important in repair processes and, finally,
- regulatory macrophages that suppress or down-regulate immune responses.

The regulatory macrophages include tumor-associated macrophages (TAM) and a subgroup of the related myeloid-derived suppressor cells (MDSC). Both play an important role in tumor progression and will be discussed later.
As for the classification by activation triggers, the authors of the classification by fundamental function also stress that hybrid and transition states exist between all populations. The classification by function, however, matches better with recent observations discussed below.

A case in point is the metabolism of the amino acid arginine by macrophages. It is often stated that the macrophage populations named above differ in their usage of the amino acid arginine, which is a substrate for both nitric oxide synthases (NOS) and arginases (ARG) (Figure 1). Expression of *Nos2* is characteristic for classically activated macrophages (or M1), whereas STAT6-dependent expression of *Arg1* (17) has always been associated with alternatively activated and regulatory macrophages (subpopulations 2a & 2c), but not macrophages activated via immune complexes and TLRs (subpopulation 2b). However, STAT6-independent induction of host *Arg1* gene expression by TLR stimulation has been demonstrated in classically activated macrophages infected with intracellular pathogens (18), such as Mycobacterium bovis or bacillus Calmette-Guérin (BCG). In addition, characterization of wound macrophages in a polyvinylalcohol (PVA) sponge wound model in mice has surprisingly revealed that wound macrophages express several markers of the alternative phenotype, including ARG1, but that neither IL4, IL13 nor STAT6 phosphorylation plays a role in the activation of those macrophages (19). We, therefore, strongly support the suggestion (8) that a classification of macrophages based on their function *in vivo* is more useful than a classification based on stimuli required for eliciting a specific macrophage subtype *in vitro*.

Arginine metabolism is not a simple marker for macrophage activation status, but has a strong effect on the outcome of many diseases. This review will, therefore, investigate macrophage arginine metabolism and its role in chronic diseases.

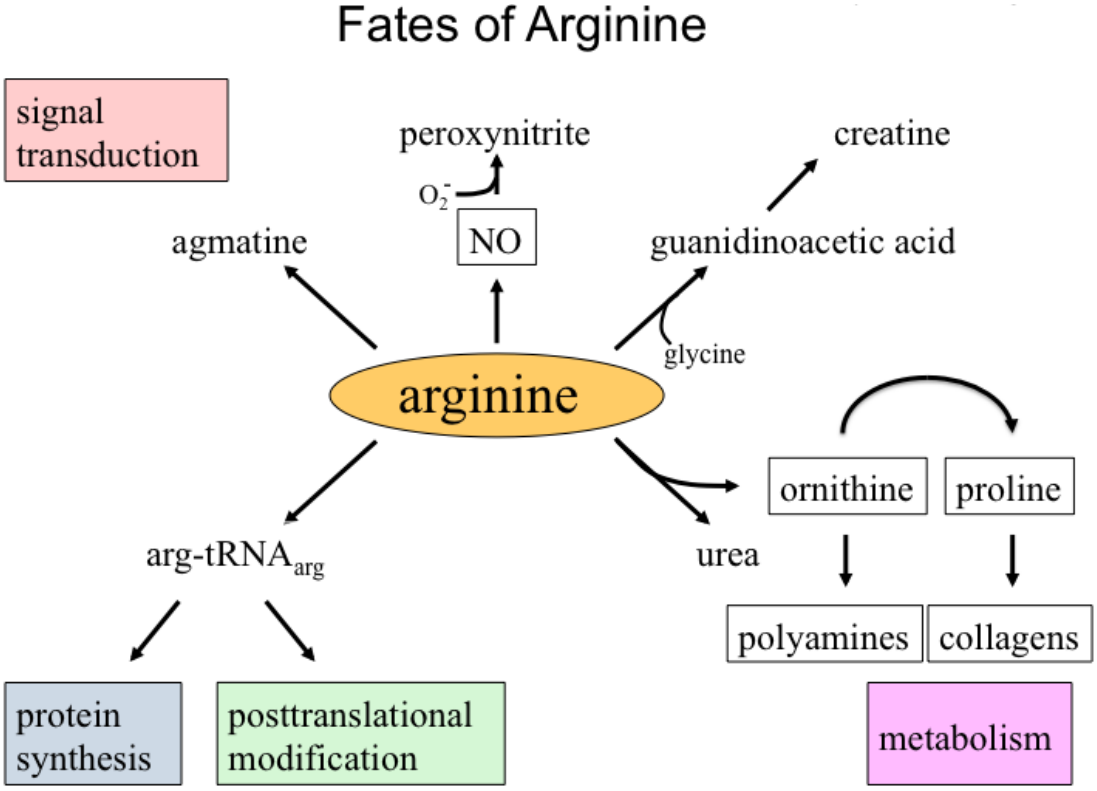

Figure 1. Fates of Arginine. Arginine is one of the 20 basic amino acids and as such a precursor for protein synthesis. Arg-tRNA also serves to arginylate proteins, an important pathway in protein degradation and protein modification during development (167). Arginine is the precursor for signaling molecules, namely agmatine, which seems to be an important neuromodulator (168) and, more importantly, the radical nitric oxide, which is generated by nitric oxide synthases and is amongst other things a relaxing agent for smooth muscle cells and a bactericidal agent in the immune defense. Arginine is also a precursor for creatine production. Arginases break down arginine to yield ornithine and urea. Ornithine is a precursor for polyamines and proline. This review focuses on two aspects of macrophage arginine metabolism that is NO formation and arginine degradation, and their consequences for health and disease.

Macrophage subpopulations

Macrophages can respond to external stimuli with dramatic changes in gene expression. Dependent on the kind of stimulus, different gene expression programs are induced resulting in macrophages with very different immunological properties. Understanding these properties and being able to modify them could lead to very promising new therapeutic approaches. A recent review suggests the use of epigenetic interventions such as targeting histone deacetylases to modulate macrophage polarization and to thereby treat inflammatory diseases (20).

Macrophages involved in host defense, or classically activated macrophages

IFNγ, together with TNFα or stimuli that induce TNFα, will result in macrophages that play a role in host defense, the so-called classically activated macrophages. These macrophages are characterized by a high capacity to present antigen, are immune effector cells that kill intracellular pathogens through NO and oxygen radical production, and secrete pro-inflammatory cytokines, such as IL1, IL6, IL12 and IL23 to induce and amplify T_H1 and T_H17-dependent immune responses (8). T_H17-cells are a group of recently identified T-helper cells associated with chronic inflammation in autoimmune diseases and host defense in bacterial and fungal infections (21) (22). In analogy with the T_H1 nomenclature, these macrophages are often called M1. They are important in host defense, but are also potent mediators in inflammatory diseases, such as inflammatory bowel disease (IBD) (23) (24) and atherosclerosis (25). If not properly controlled, they may also play a role in cancer development through persistent inflammation (26). Classical activation in murine macrophages leads to the induction of NOS2 and cationic amino-acid transporter 2B (*Cat2b or Slc7a2b*) expression (27,28), which results in increased arginine uptake and NO production.

Alternatively activated macrophages or wound healing macrophages

Alternatively activated macrophages, a subgroup of the M2 macrophages, are induced *in vitro* by IL4 and IL13 with enhancing roles suggested for IL21 (29), IL33 (30,31) and galectin-3 (32). The cytokines IL4 and IL13 signal via STAT 6 and this results in the expression of typical marker genes, such as *Arg1*, the macrophage mannose receptor (mannose receptor, C type 1 (*Mrc1*)), resistin-like β (*Retnlb*, also known as *Fizz1*), and chitinase3-like protein3 (*Chi3l3*, also known as *Ym1*), and, at least in culture, *Cat2b* (*Slc7a2b*) (28). Note that *Cat2b* is also a marker for classically activated macrophages. These macrophages are thought to promote wound healing through extracellular matrix reorganization and stimulation of fibroblasts to increase collagen production. This is reflected in their expression of growth factors that promote fibroblast proliferation and proteins that interact with, or support extracellular matrix deposition. It is often suggested that the role of arginase 1 in this scenario is the production of ornithine, which can serve as a precursor for either proline or polyamines, relevant molecules in collagen production and cell proliferation, respectively (8).

A more detailed *in vivo* characterization of wound macrophages in a murine PVA sponge model now challenges, however, the concept that the alternatively activated macrophages of the Petri dish are the wound-healing macrophages encountered *in vivo* (19). Wound macrophages isolated on days 1, 3 and 7 from sponges implanted subcutaneously in mice indeed express arginase 1, CHI3L3, mannose receptor and TGFβ proteins, but they also produce TNFα, a marker for classically activated macrophages. More importantly, neither IL4/IL13 nor STAT6 phosphorylation seem to be involved in eliciting the expression of these marker genes. IL4 or IL13 were not found in wound fluid, wound macrophages showed no STAT6 phosphorylation and,

most compellingly, *Il4ra*$^{-/-}$ macrophages, which lack functional IL4 and IL13 receptors, are indistinguishable from wild type wound macrophages in this model. The lack of IL4 in wound fluids was previously demonstrated in rodents in a burn wound model (33) and in foreign body-response models (34,35), lending extra credibility to the findings of Daley et al. (19,36). The absence of IL4/STAT6 signaling has recently also been confirmed in wound-healing macrophages in a laser-injury wound model of choroidal neovascularization in the eye (37).

From these data, we conclude that wound-healing macrophages express the markers described for alternatively activated markers, but can obviously be induced by stimuli other than IL4 and IL13. Depending on the type of wound, the same macrophage function can be elicited by different triggers. In helminth infections, IL4 and IL13 are abundant and expression of wound healing markers is STAT6 dependent (38). Other types of wounds, such as sterile foreign body-induced wounds or burn wounds, may depend on other triggers to activate the desired macrophage phenotype. Again, this argues for a classification of macrophages by function rather than by external stimuli. The question that remains, however, is – what is the trigger for the wound healing phenotype in sterile wounds? One good candidate for such an alternative trigger is TGFβ that is found in abundance in certain wound fluids and capable of inducing arginase 1 protein expression (39) in fibroblasts (40) and microglia (41), and increasing arginase activity in macrophages (42).

Regulatory macrophages or all other macrophages?

The term regulatory macrophages was introduced by David Mosser and Justin Edwards (8) to distinguish macrophages with strong anti-inflammatory activity from wound-healing macrophages. These regulatory macrophages are characterized by high IL10/low IL12 expression and are elicited by various stimuli including immune complexes that bind to TLRs, prostaglandins, G-protein coupled receptor (GPCR) ligands, glucocorticoids, apoptotic cells or by IL10 stimulation. They also express *arginase 1* mRNA (43), although ARGINASE 1 protein expression has been described as limited (44) or is not mentioned at all (45). In addition, they produce the chemokines CCL17, -18 and -22, and also TGFβ (4,5). IL10 production is high in TLR-stimulated macrophages, whereas stimulation by IL4 and/or IL13 alone (definition of alternatively activated macrophages) does not seem to result in significant IL10 expression (8), although conflicting reports exist (5,46). Unfortunately, high IL10/low IL12 expression is also named as a characteristic of tumor-associated macrophages and myeloid-derived suppressor cells (discussed below in "Arginase as a controller of arginine availability" and "Arginase and Cancer"), but often also simply for M2 macrophages (47) to distinguish them from M1 macrophages (5,48), which renders this characteristic useless for a meaningful distinction of macrophage populations. In agreement, a recent article on the nomenclature of macrophages started with the encouraging sentence: "Description of macrophage activation is currently contentious and confusing" (22). The authors strongly advise a coherent nomenclature based on macrophage origin, activators

used and a consensus set of markers for characterization. As stated above, however, a nomenclature based on these criteria is most useful for *in vitro* experiments, where the activators can be controlled.

It is very obvious that macrophages can be activated by a wide variety of triggers on the one hand and can respond with an equally wide range of activation profiles on the other hand: the more markers are measured, the more different populations are found. The usefulness of these distinctions is mostly descriptive and depends on the experimental circumstances. The distinction of regulatory and wound-healing macrophages is based on the assumption that wound-healing macrophages participate, in addition to exerting their immunosuppressive activities, also in matrix deposition and possibly tissue healing/proliferation through ornithine production by arginase 1. Ornithine can serve as a precursor for both proline and polyamines (Figure 1). Recent research, however, seems to indicate that there are only two main functions of macrophages, namely immune activation and immune suppression and that the arginase activity of wound-healing macrophages is more important in immune suppression than in matrix deposition or proliferation. This will be discussed below.

Arginine metabolism of macrophages

Arginine transport in murine macrophages

Murine macrophages take up arginine via the y^+L or y^+ system of transporters. The y^+L system is well characterized (49) and consists of a light chain (SLC7A6, a.k.a y^+LAT2, or SLC7A7, a.k.a y^+LAT1; SLC stands for solute carrier family) that associates with a heavy chain (SLC3A2, a.k.a 4F2hc or CD98) for surface expression. These heterodimeric transporters are exchangers, which are subject to "trans-stimulation". Trans-stimulation occurs when a compound on one side of a membrane stimulates transport on the other side of the membrane (50). y+L transporters preferably import a large neutral amino acid (such as leucine) plus Na^+ in exchange for the export of a cationic amino acid. y^+LAT2 can mediate an efficient influx of large neutral and cationic amino acids, but the efflux is clearly biased towards cationic amino acids. The uptake of arginine into cells is, therefore, often inhibited with leucine *plus* sodium, which actually stimulates arginine efflux. In the absence of extracellular leucine, measurable arginine y^+LAT2-mediated influx occurs, as was shown in erythroid cells (51).

The y^+ system is encoded by the cationic amino-acid transporter (*Cat*) genes *Slc7A1* (*Cat1*), *Slc7A2* (splice variants *Cat2a* and *Cat2b*) and *Slc7A3* (*Cat3*). These transporters all mediate Na^+-independent transport of the cationic amino acids arginine, lysine and ornithine (52,53), but CAT1 shows a strong trans-stimulation, which is lacking in CAT2. The three transporters differ in spatial and temporal expression patterns. CAT3 is important in embryogenesis and is found in central neurons in the adult. *Cat1* mRNA is often constitutively expressed, but its translation (54) and activity (55) are strictly regulated by nutrient status, cytokines and hormones. CAT2 exists in 2 splice-variants.

The constitutively expressed, low-affinity CAT2A variant is expressed in liver, pancreas, skeletal muscle, cardiomyocytes and vascular smooth muscle, while the high-affinity CAT2B is inducible by inflammatory cytokines.

Under basal, i.e. non-stimulated conditions, macrophages take up most of the arginine via the y^+L system. Once they are stimulated with M-CSF to proliferate, increased arginine import via CAT1 is observed (28). Stimulation of macrophages with either the T_H1 cytokine IFNγ plus LPS or the T_H2 cytokines IL4 and IL10 leads to upregulation of *Slc7a2b (Cat2b)* (28). *Cat2b* expression stimulated by T_H1 cytokines is often induced together *Nos2* expression. Accordingly, sustained NO production by peritoneal macrophages from *Cat2*$^{-/-}$ mice is virtually abolished (56). Intracellular arginine concentration and *Nos2* expression, however, are unchanged in these macrophages. This has been interpreted as a strong indication for the presence of different arginine pools in macrophages that are not freely exchangeable (49). Another study in experimental asthma showed upregulation of *Cat2* together with *Arg1*, but not *Nos2*; unfortunately, the *Cat2* isoform that was expressed (A or B) was not identified (57).
Based on a study on murine bone marrow-derived macrophages with ^{14}C-labeled arginine (28), the following model was suggested: quiescent macrophages import arginine via the y^+L system, incorporate a small amount thereof into proteins and return the rest to the medium. Proliferating macrophages, stimulated by M-CSF, probably take up arginine through CAT1. 50% of this arginine goes into protein synthesis, 5% into polyamine synthesis and the rest is returned to the medium. Classically or alternatively activated macrophages both import arginine via CAT2B and use it for NO production via NOS2 or for ornithine/urea production via arginase 1, respectively (28). However, data from *Cat2*$^{-/-}$ mice support those findings only partially. Infection of such mice with Toxoplasma gondii, which elicits a T_H1-type response, indeed results in a much higher susceptibility to the infection due to decreased NO production. Infection of the same mice with Schistosoma mansoni, which results in a pronounced T_H2 response, also led to the development of more severe disease-related pathologies. However, bone marrow-derived macrophages from these mice show higher urea production, a measure of arginase activity, in culture, when stimulated with T_H2 cytokines. In the same study, higher arginase 1 mRNA expression was found in lung tissue of infected mice (58). These observations strongly suggest that arginase 1 also has access to a CAT2-independent arginine pool, but the experiments performed do not allow the distinction between an extracellular or intracellular source of arginine. Again, it is obvious that the association of specific stimuli with specific transporters found in vitro cannot be directly translated into the in vivo situation.
A recent study (24) demonstrated that the different susceptibility of C57BL/6 and Balb/C mice to Leishmania infections is due to a mutation in the *Cat2* promoter of C57BL/6 mice, which results in diminished arginine uptake into macrophages when stimulated with IL4 (but not IFNγ). Increased CAT2 activity provides arginine for polyamine synthesis for the intracellular pathogen in Balb/C, but not in C57BL/6 mice.

Neither *Cat1* (y^+) mRNA expression nor basal y^+L-mediated arginine transport are affected by macrophage activation (28). Since CAT1 protein expression is regulated at the translational rather than the transcriptional level in mice (54) and men (59), protein expression may still be increased and serve as a source of arginine in T_H2-cytokine-stimulated macrophages. The lack of a specific antibody has been hampering a detailed investigation of the regulation of *Cat1* under these circumstances. Unfortunately, *Cat1*$^{fl/fl}$*/Tie2Cre* mice on a *129Sv/OlaHsd* background do not survive after birth due to severe anemia, similar to the phenotype of constitutive *Cat1*$^{-/-}$ mice [our own unpublished observations: CC de Theije, SE Köhler, WH Lamers]. Hopefully, *Cat1*$^{fl/fl}$*/LysMCre* mice will enable us to address the role of CAT1 in substrate delivery to macrophages under type II inflammatory conditions. Intriguingly, backcrossing the *Cat1*$^{fl/fl}$*/Tie2Cre* from a129Sv/Ola background to C57BL/6J increases the life expectancy of these mice to adult age and even allows the breeding of homozygous mice. They remain, however, fragile with a higher than normal mortality and smaller than their wild-type littermates as adults, which hampered vessel cannulation for blood-pressure measurements [our own unpublished observations: CC de Theije, SE Köhler, WH Lamers, R. Chennupati].

Arginine transport in human macrophages

Arginine transport in human monocytes and macrophages is less well characterized than in mice. System y^+L is reportedly mainly responsible for basal transport in human alveolar macrophages. These cells do not respond with increased arginine import or NO production to LPS stimulation, although they upregulate *CAT2B* mRNA expression (60). In contrast, an increased y^+-dependent transport activity and NO production was reported in PBMCs from septic patients (61). The authors assigned the increased transport to CAT2 expression, since they found *CAT2* mRNA in 5 of 7 PBMC samples from septic patients, but not in controls, and no change in *CAT1* mRNA levels in the two groups. It is possible, however, that translation of *CAT1* mRNA was also upregulated in these patients. This hypothesis is supported by an increased NO production without concomitant *CAT2* mRNA expression in 2 of the 7 septic patients. Increased *CAT2* expression and y^+-dependent arginine transport have also been reported in PBMCs of pregnant women, but no clear correlations between arginine transport, *CAT2* expression and NO production were found (62). Stimulation of freshly isolated human monocytes with IFNγ resulted in increased arginine import attributable to system y+L and was accompanied by an increased expression of *SLC7A7* (y+LAT1) (63), whereas mRNA expression of other cationic amino-acid transporters such as *CAT1* (*SLC7A1*) and *CAT2B* (*SLC7A2*) was unaffected. A more rigorous analysis of arginine transport in human macrophages is highly desirable.

In summary, data from murine macrophage cultures suggest that activation of macrophages leads to stimulation of arginine transport through gene and protein expression and that the same transporter, CAT2B, is used in both the classical and alternative activation state (28,58). *In vivo* data with macrophage-specific gene deletion

confirmed the role of CAT2 under T_H1 conditions, but were less convincing for T_H2 conditions. The question whether the same intracellular arginine pool is accessible for NOS2 and arginase 1 is, therefore, still not fully answered. The fact that *Cat2*$^{-/-}$ mice are viable and fertile suggests that there is redundancy in arginine transporters in C57BL/6J, but probably not in 129Sv/Ola mice. Moreover, the metabolic activity and intracellular arginine content of *Cat2*$^{-/-}$ macrophages is not affected in the basal, unchallenged state (56), a finding that fits with the observation that the system y^+L is the major arginine transporter under those conditions (28).

It is tempting to speculate that the different degrees of trans-stimulation observed for CAT1, CAT2A and CAT2B add an extra level of control. In such a scenario, CAT1 would serve as the ideal transporter in an arginase 1-expressing cell to deplete the environment of arginine, CAT2B would be best for arginine uptake in a cell that rapidly metabolizes arginine, and CAT2A would allow arginine uptake and accumulation without (excessive) export of another cationic amino acid in exchange. The expression of CAT2A in liver cells and CAT2B in activated macrophages fits with this suggestion, but the role of CAT1 as the transporter of choice in proliferating macrophages is difficult to combine with its pronounced exchanger profile.

Control of arginase expression in murine macrophages

Arginase 1 expression is constitutive in periportal hepatocytes, but inducible by cytokines in other cell types, such as macrophages and fibroblasts. The majority of extra-hepatic arginase 1-expressing cells are macrophages and dendritic cells as shown by a reporter mouse that carries an IRES-YFP knock-in allele into the 3'-end of the arginase 1 gene (64). In macrophages and dendritic cells, arginase 1 expression is associated with T_H2-type cytokines, such as IL4, IL10, IL13 and TGFβ, which are all strong inducers of arginase 1 expression *in vitro*. This induction is STAT6- and C/EBPβ-dependent (65). Remarkably, in classically activated macrophages, STAT6-independent, MyD88- and C/EBPβ-dependent expression of arginase 1 has been demonstrated after infection with intracellular pathogens (18).

Mouse models, deficient for IL4, IL10, IL13 or combinations thereof indicate that IL4 and IL13 are the major inducers of arginase 1 expression *in vivo*. IL10 (66) shows significant synergistic effects with IL4 on arginase 1 induction, probably due to the upregulation of *Il4ra* expression (67). On its own, IL10 is only able to induce a low level arginase 1 expression.

Control of arginase 1 expression in human macrophages or myeloid cells

The expression of arginase 1 in human macrophages is still controversial. Several studies have shown that in human monocytes/macrophages *in vitro* alternative activation by either IL4, IL10 or IL13 does not lead to induction of arginase 1 (and other murine markers of alternative activation) (68-72). Other studies, however, have shown that

human macrophages can and do express arginase 1 during filarial infection *in vivo* (73) and after infection with *Coxiella burnetti in vitro* (74). The same authors demonstrated that uptake of apoptotic cells induced an alternative phenotype in human macrophages characterized by high expression of mannose receptor and secretion of TGFβ and IL10. Neutralization of IL10 or TGFβ prevented persistent Coxiella infection (75). Interestingly, both types of infection also result in alternative macrophage activation in susceptible mouse strains (76,77). In addition, weak arginase 1 gene expression has been shown in human adipose-tissue macrophages (78). Again, this provides an argument for classification of macrophages by function (i.e. the protein expression profiles) rather than activation trigger *in vivo*. For *in vitro* studies, the recently proposed, more complex nomenclature framework for macrophages, which is based on the source of the macrophages, the activators and a consensus selection of markers, will be useful for comparison of different cell populations and cell populations of different research groups (22).

Immune functions that are covered by macrophages in mice may be performed by other immune cells in humans. Constitutive *ARG1* expression is found in human circulating PMNs or granulocytes, which sequester arginase 1 in intracellular granules. Upon certain triggers, these granules are released into plasma and decrease arginine concentration there (69,79,80).

Control of arginase expression in other cell types

TGFβ-dependent induction of arginase 1 has also been shown in vascular smooth muscle cells and resulted in measurable increases of putrescine and proline production *in vitro* (81). TGFβ also seems to be an important inducer of arginase 1 in fibroblasts (82,83). In addition, arginase 1 expression was shown in endothelial and vascular smooth-muscle cells of aged rats (84,85) and in human intestinal microvascular-endothelial cells after stimulation of with the inflammatory triggers LPS and TNFα (86). Wound fibroblasts express high levels of arginase. Interestingly, deletion of CAT2 leads to increased arginase activity in both cultured macrophages and fibroblasts as determined by urea production (58). The authors suggest that CAT2 suppresses arginase activity, but the mechanism is unclear. The role of CAT2B upregulation by type II cytokines would then be limitation of arginase activity rather than provision of substrate, thereby limiting the extent of type II response-associated inflammation.

Arginase as a controller of arginine availability

Intracellular arginase 1 expression can control local extracellular arginine levels and, thus, reduce arginine availability for neighboring cells that do not express arginase. The suggested mechanism involves rapid uptake of arginine by a cationic amino-acid transporter (CAT) and degradation by cytosolic arginase 1. The formed ornithine, in turn, stimulates arginine import via CAT-mediated ornithine-arginine exchange. This mechanism seems highly relevant in immunosuppression, where alternatively activated

macrophages or related cells, such as dendritic cells or granulocytes, can suppress T-cell activation by local arginine depletion. T-cells are very sensitive to arginine deficiency, as has been demonstrated both *in vitro* in murine (87,88) and human cells (89,90), and *in vivo* in mouse tumor models (91,92).

The so-called myeloid-derived suppressor cells (MDSC), a collective name for cells that support tumor growth in murine models and in human cancers, and includes precursors of granulocytes, macrophages, dendritic cells, or early myeloid progenitors. MDSCs express arginase 1 intracellularly (93) and use CAT1-mediated ornithine-arginine exchange to locally deplete the extracellular fluid of arginine through uptake and subsequent degradation to ornithine and urea (88,94). Ornithine may be further metabolized to polyamines or exported. As a consequence of arginine deficiency, T-cells become anergic through the activation of GCN2 kinase (95), which results in suppression of general protein translation. *In vitro*, this process of T-cell inhibition can be reversed through the addition of arginine.

It has been suggested, that cells expressing the cationic amino-acid transporter CAT1 may even be depleted of their intracellular arginine, when the cells are in contact with extracellular arginase or an arginase-expressing cell (96). This depletion results from trans-stimulation of CAT1-mediated arginine export from the cell by another extracellular cationic amino acid, because CAT1 functions much more efficiently as an amino-acid exchanger than as a unidirectional transporter (see section on "Arginine transport"). High extracellular concentrations of ornithine provided by arginase activity in another cell, i.e. a MDSC, or by extracellular arginase activity could thus induce increased ornithine import in exchange for arginine export from neighboring cells, such as T-cells, thereby rapidly depleting those cells of arginine and making them anergic (95,97). Once depleted, CAT1-expressing cells take up arginine only very slowly. Although this sounds like a very elegant and plausible mechanism, CAT1 expression in lymphocytes has, as far as we know, not yet been demonstrated.

Arginine – Proline

Arginine can also serve as a precursor for proline or glutamate, provided ornithine aminotransferase (OAT) is co-expressed with arginase. In this case, ornithine produced by arginase is converted by OAT to glutamic γ-semialdehyde, which cyclizes non-enzymatically to pyrroline-5-carboxylate, which is then reduced by NADPH-dependent pyrroline-5-carboxylate reductase to proline. It has been repeatedly suggested that arginase expression acerbates fibrosis due to excessive collagen production from proline (98-100) or supports wound healing through the same mechanism (101). M2 macrophages of the “wound-healing” type have in particular been associated with increased production of proline in fibrotic disease (102). Arginase 1-overexpressing mice, however, show no fibrosis in any tissue and certainly not in the gut where the enzyme is overexpressed (103,104). Few papers show actual experimental data linking arginase expression to proline formation or collagen deposition. Dependence of collagen

deposition on arginase activity was shown in murine fibroblasts *in vitro* and in the same paper increased arginase 1 expression was demonstrated in myofibroblasts in bleomycin-induced pulmonary lesions in mouse lung (40). Arginase-dependent proline production has also been shown in VSMC stimulated by either cyclic stretch or TGFβ1 (81,105). Proline production from arginine was demonstrated *in vivo*, but the amount of arginine-derived proline incorporated into protein-bound proline was only 5% of total that of direct proline incorporation (106), indicating that this metabolic route is hardly relevant. The *in vivo* finding that proline is abundant in wound fluid and arginine scarce (in rodents) further supports the notion that arginine is not an important precursor for collagen synthesis, even though both arginine and ornithine have positive effects on hydroxyproline production and scar strength in mice (107). Interestingly, the stimulatory effect of arginine (108), but not that of ornithine (109) on hydroxyproline production and scar strength are abolished in $NOS2^{-/-}$ mice, suggesting that the NOS2 pathway plays a role in mediating the effects of arginine on wound healing. In cell culture, inhibition of arginase reduces collagen synthesis in rat, but not in human fibroblasts (82). Since inhibitor studies may be hampered by undesired inhibition of enzymes other than the intended target, results from arginase knockout mice can hopefully further clarify the issue. A recent study by Campbell et al. (37) indeed showed that arginase 1 is required to accelerate wound healing, since absence of arginase 1 from macrophages resulted in delayed wound closure and a more inflammatory wound environment. The absence of arginase 1 in this model resulted in increased numbers of NO-producing cells, but did not diminish collagen expression. This however did not result in increased matrix deposition, since the expression of gelatinases (matrix metalloproteinases) was also increased in this pro-inflammatory environment. Again, the main effect of arginase seems not to lie in the provision of precursors for proline and hence collagen synthesis, but rather in the control and suppression of excess inflammatory cells.

Lessons from Schistosoma infections

Mice deficient in M2 macrophages due to a macrophage-specific disruption of the IL4Rα gene (Interleukin-4 receptor alpha chain) show no change of fibrotic lesion size after Schistosoma mansoni infection. These mice do, however, succumb to the disease much faster than their wild-type littermates due to excessive T_H1 cytokine responses and increased NOS2 activity (110). In a less drastic mouse model with macrophage-specific deletion of arginase 1 (*Arg1*$^{fl/fl}$*/Tie2-Cre* and *Arg1*$^{fl/fl}$*/LysM-Cre* mice with arginase 1 deletion in all hematopoietic and endothelial cells, or in granulocytes and macrophages, respectively), infection with Schistosoma mansoni resulted in even larger fibrotic lesions in the knockout animals than their arginase 1-expressing litter mates (92). These findings suggest that macrophage arginase 1 protects against rather than sensitizes to fibrosis. Furthermore, the local depletion of arginine by alternatively activated macrophages suppressed proliferation of T-cells in this model. A consequence of this suppression was a reduction of excessive macrophage accumulation in granulomatous lesions. This suggests that the major role of macrophage arginase 1 is suppression of T-cell proliferation by local arginine depletion. The observed increased fibrosis in the

arginase-deficient model is probably a consequence of increased stimulation of collagen deposition by myofibroblasts through inflammatory cells. A very recent review by Wynn & Barron (111) also emphasizes the role of macrophages as regulators of fibrosis controlling the collagen-depositing activity of myofibroblasts. These authors also interpret the role of macrophage arginase as a controller of arginine availability, depriving both $CD4^+$ T-cells and myofibroblasts of a metabolite that is essential for their survival. Supportive evidence for this claim comes from Schistosoma-infected *Cat2*$^{-/-}$ mice that show increased arginase 1 expression in myofibroblasts in granulomatous lesions with concomitant collagen deposition and increased lesion size (58). The ornithine produced by alternatively activated macrophages obviously played no significant role in stimulating collagen deposition, whereas the depletion of arginine by macrophages reduced arginine availability to myofibroblasts. There are two possible explanations for these seemingly contradictory findings. Firstly, extracellularly produced ornithine is not a precursor for collagen, whereas intracellularly produced ornithine is, or secondly, ornithine in general is not a relevant precursor for collagen-incorporated proline. Two studies that investigated the role of arginine in wound healing come to a similar conclusion, namely, that it remains to be proven that ornithine is an important precursor of collagen-incorporated proline (106,107).

Arginine – Polyamines

Polyamine function

Polyamines, such as putrescine, spermine and spermidine, are aliphatic cationic molecules that consist of, respectively, 1, 2, and 3 putrescine moieties. Due to their highly positive charge, polyamines bind and stabilize RNA and DNA. Moreover, they seem to be able to regulate local histone acetylation via upregulation of histone acetylases (HATs) and histone deacetylases (HDACs) (112). They can increase the accessibility of promoters and facilitate transcription and translation of specific genes (for a review see (113)). Polyamines are also thought to play an important role in cell cycle regulation and proliferation (114). Liver regeneration after partial hepatectomy is, for example, absolutely dependent on the presence of spermidine (115,116). Furthermore, polyamine metabolism is frequently deregulated in cancer (117). Accordingly, polyamine levels are tightly controlled by feedback mechanisms that regulate synthesis, breakdown and transport. These feedback mechanisms result in adaption of the expression of polyamine-synthesizing or -degrading enzymes (118,119) to keep free polyamine concentrations stable (Figure 2).

Polyamines are either taken up from the diet or synthesized by decarboxylation of ornithine by ornithine decarboxylase (ODC), which catalyzes the first, rate-determining step. Decarboxylation of arginine to agmatine can also, via agmatinase, yield putrescine. Agmatinase is structurally related to arginase. Both arginine decarboxylase and agmatinase are expressed in RAW 264.7 macrophages and this expression is regulated by LPS, TGFβ, IL4 and IL10 (120). However, there is some doubt about the

functionality of murine agmatinase (121) Most of the evidence for the requirement of polyamines for cell function comes from experiments using the inhibitor α-difluoromethylornithine (DMFO or eflornithine), and knockout or overexpression studies of ODC (117).

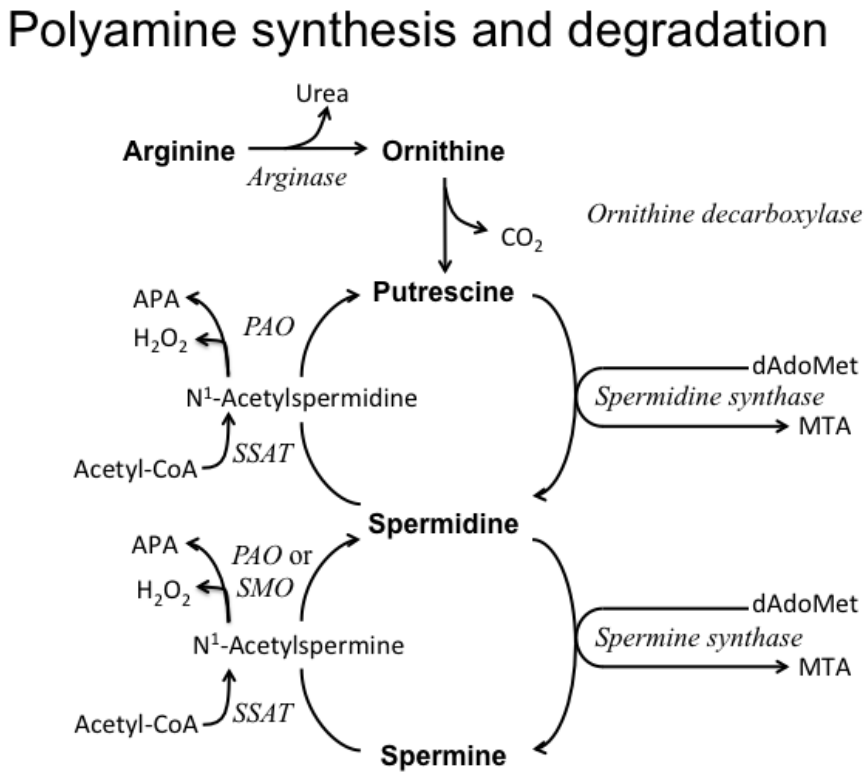

Figure 2. Polyamine Synthesis and Degradation. Synthesis and degradation of the three polyamines putrescine, spermidine and spermine and the most important enzymes and precursors or degradation products in shown. APA: acetamidoproprionaldehyde, dAdoMet: decarboxylated S-adenosylmethionine, MTA: methylthioadenosine, PAO: polyamine oxidase, SMO: inducible spermine oxidase, SSAT: Spermidine/spermine-N^1-acetyltransferase

Arginase and polyamine synthesis

Since arginine is a precursor for ornithine, arginase activity can exert control over polyamine synthesis when ornithine is limiting, as has been shown in experiments where arginase 1 was either overexpressed (122) or induced by TGFβ in vascular smooth muscle cells (VSMC) (81); see also (123) for references). Since polyamine production and ODC expression are elevated in many tumors (118) and in wound healing (124), and since arginase 1 expression has also been associated with tumor growth and metastasis (125), and wound healing (126), the logical conclusion seems that arginase 1-expressing macrophages may increase polyamine production to support tissue regeneration in wounds (127) and vascularization of tumors (128). Experimental evidence for this particular hypothesis is, however, scarce. Thus far, few papers have investigated changes

in expression and /or activity of both ODC and arginase simultaneously. A study on arginine- and ornithine-metabolizing enzymes in the kidney after testosterone treatment (129) showed a 1.5-fold increase in arginase activity and a 450-fold increase in ODC activity. Since incorporation of the carbon backbone of arginine into polyamines was not determined, it is precarious to deduce a functional relation. A similar critique can be raised against a recent study (130), in which the stimulatory effects of smoking in asthmatics on arginase and ODC expression were reported. In this study, enzyme activities did, for instance, not correlate with polyamine concentrations or levels of polyamine-degrading enzymes. Similarly, a recently claimed positive correlation of polyamine concentrations and arginase activity in sputum was not convincing, see below (16).

Another compelling example arguing against a direct relation between arginase levels and polyamine synthesis is that arginase 1 and arginase 2 single- and double-knockout mice show hardly any changes in polyamine levels in liver, kidney, small intestine or brain, even though the animals were only 2 weeks old (131). Similarly, polyamine levels (putrescine, spermine and spermidine) in the small intestines of mice overexpressing arginase 1 in their enterocytes were not increased, despite a 30% increase in ornithine in the same tissue (103). Finally, IL4 induced polyamine production is not impaired in arginase 1-deficient macrophages (15). On the contrary, *Arg1*-deficient macrophages even showed increased polyamine production upon IL4 stimulation when compared to their wild-type counterparts. A possible explanation for this surprising finding is that, instead of providing ornithine as a substrate, arginase 1 activity, together with a cationic amino-acid exchanger, depletes the cell of ornithine (for each molecule of arginine imported, one molecule of ornithine is exported), thereby efficiently preventing polyamine synthesis. In the absence of arginase, ornithine is no longer exported and available for ODC. Together, these findings suggest that arginase 1 suppresses polyamine synthesis in the expressing cell, i.e. the macrophage. In support, a recent study in mice on the effects of polyamines on asthma showed virtually no correlation between increased concentrations of polyamines and arginase activity in sputum (16). Polyamine concentration was 1.5-fold increased (with very little variation) in this model. Arginase 1 activity was, as expected, not detectable in non-asthmatic sputum and elevated in asthmatic sputum, but the variation in arginase 1 activity was no less than 6-fold.

Polyamine flux

It was recently shown that even though tissue polyamine concentrations do not change when ODC or SSAT are overexpressed or inhibited/knocked-out, polyamine turnover and thus metabolic flux may change considerably. Increased polyamine flux can lead to the depletion of important metabolites, such as cytosolic acetyl-CoA and malonyl-CoA (132) (see Figure 2), and to an increase in toxic by-products such as H_2O_2. It is, therefore, possible that unchanged polyamine levels mask an increased polyamine flux, in particular when substrate levels are high. Some of the phenotypic abnormalities observed in arginine-deficient mice, such as hair loss in arginase1-overexpressing F/A2

mice and ornithine transcarbamoylase-deficient Spf/Ash mice (133) are comparable to those of spermidine/spermine N1-acetyltransferase (SSAT)-overexpressing mice. Overexpression of SSAT results in an increased polyamine flux, but is also characterized by an accumulation of putrescine, which was not found in F\A2 mice. Circumstantial evidence in the SSAT mice points to putrescine accumulation as the culprit of the hair loss (see Minireview (115)), whereas in F/A2 mice, tissue polyamine levels were not changed. Instead, the arginine deficiency had activated the integrated stress response kinase GCN2 in many tissues (134). A recent review (135) presents evidence that polyamines can induce stress responses resulting amongst other things in inhibition of general protein synthesis, similar to the effects of GCN2-mediated eIF2α phosphorylation in mammals. Whether hair loss in transgenic SSAT, Spf/Ash, and F\A2 mice is the consequence of activation of the integrated stress response is intriguing and warrants further investigation.

Polyamine synthesis in macrophages

The increased polyamine synthesis in VSMC cells that overexpress arginase 1 (see section on "Arginase and polyamine synthesis") could not be reproduced in the murine macrophage cell line RAW 264.7, suggesting that arginase-derived ornithine is only rate-limiting for polyamine production when no other ornithine source such as proline or glutamate (and the necessary enzymes) is available (136). An arginase-dependent increase in polyamine synthesis in RAW 264.7 cells could, nevertheless, be demonstrated after LPS- or 8-Bromo-cAMP-stimulated ODC expression. After 6 hours of treatment with LPS or 8-Bromo-cAMP stimulation, arginase 1-overexpressing macrophages showed marked increases in the levels of putrescine (3-fold) and spermidine (1.5-fold) compared to lacZ-transfected or untransfected control cells. The higher increase in putrescine than in spermidine is in line with increased polyamine flux (115) in these cells to maintain stable polyamine levels and is not accompanied by increased cell growth (136).

The experiment described above mimics the situation in pathogen-infected macrophages. Indeed, increased polyamine production in macrophages is observed *in vivo* after infection with different pathogens, and this increased production has disastrous effects on the macrophages themselves. The opportunistic pathogen Pneumocystis induces upregulation of AZI, the antizyme inhibitor of ODC antizyme, which results in increased cellular ODC protein and polyamine levels. This, in turn, results in increased polyamine oxidase expression and activity. A by-product of polyamine oxidation is H_2O_2, which leads to macrophage apoptosis (137). Helicobacter pylori infection has a similar effect via a different mechanism. Helicobacter activates c-Myc in gastric macrophages, which upregulates ODC expression and spermine production. Spermine suppresses NOS2 transcription, which prevents an effective immune response against the pathogen. In addition, Helicobacter infection results in increased SMO expression (a spermine-specific polyamine oxidase; see Figure Polyamine Metabolism) and H_2O_2 production in the macrophages and concomitant macrophage apoptosis.

Susceptible ("non-healer") strains of mice, such as BALB/c, respond to Leishmania major infection with a polarized T_H2 response with increased arginase 1 expression and a concomitant increase in polyamines (138). Inhibition of arginase reduces polyamine production and parasite growth. The authors conclude that the pathogens exploit ornithine and polyamine production by the host for their own multiplication. Accordingly, non-susceptible ("healer") strains, such as the T_H1-biased C57Bl/6 mice, do not express arginase in their macrophages at the onset of infection and are therefore able to resolve it (139). Interestingly, when a healer strain is treated with a daily dose of ornithine or putrescine during Leishmania infection, the lesion size increased, lending further support to the conclusion that the pathogen uses host ornithine for growth (140,141). In the first two cases, this leads to macrophage apoptosis thereby supporting the infection, whereas in the latter case, the accelerated growth of the pathogen is supported through host polyamines. ODC inhibitors such as DMFO are already successfully used against Trypanosoma infections and other ODC inhibitors seem promising in treating Leishmaniasis (142).

In conclusion, macrophage arginase 1 can support increased polyamine synthesis in disease or infection if ODC levels are also upregulated, but this results in either host-cell apoptosis or increased pathogen growth rather than host tissue proliferation. There is evidence that fibroblast or smooth-muscle arginase 1, on the other hand, does support host cell proliferation (122,143) and tissue remodeling and that this may become excessive when not controlled by macrophage arginase 1.

Arginase and Cancer

Tumor-associated macrophages (TAMs) or myeloid-derived suppressor cells (MDSCs)

Increased expression of arginase 1 in MDSCs (93) (for a recent review see (144)) has been demonstrated in mouse models (145-147) and human MDSCs isolated from melanoma, small cell lung cancer and renal carcinoma patients (97,148,149). The effect of arginase expression, however, can either be immunosuppressive and thus tumor-promoting, or tumor-inhibiting, depending on tumor properties and arginase concentration.

Arginase 1 expression can result in local arginine depletion, which negatively affects T-cell proliferation and activation (see Arginase as a controller of arginine availability), and thereby promotes tumor growth through immunosuppression, or cancer cells themselves and thereby inhibits tumor growth. Cancer cell lines that are sensitive to arginine deprivation lack argininosuccinate synthetase (ASS), the enzyme that catalyzes the rate-limiting step in arginine synthesis from citrulline (150). Immunohistological characterization of tumors is, therefore, necessary to identify tumors that may be targeted by arginine deprivation (151,152). Interestingly, reduced ASS expression in osteosarcoma is associated with a higher risk of pulmonary metastasis after surgery (153).

Arginase expression in MDSC is, therefore, presumable only beneficial for the host when the tumor in question is susceptible to arginine deprivation, a hypothesis that can be tested in murine tumor models with arginase 1-deficient MDSC. On the other hand, increased tumorigenesis has been associated with disturbed polyamine metabolism due to elevated ODC levels and concomitantly increased polyamine concentrations (112,117), but also with increased expression of the polyamine-degrading enzyme spermidine/spermine N1-acetyltransferase (SSAT) (154). Both observations together have led to the often cited hypothesis that arginase 1-derived ornithine serves as a precursor for polyamines, which, in turn, are essential for rapidly growing tumor cells. Inhibition of arginase by N-hydroxy-L-arginine (NOHA) in susceptible tumor cells reduced spermine content and subsequently caused apoptosis (155). The same authors demonstrated later, however, that apoptosis was not a consequence of polyamine depletion but an effect of NOHA that was independent of polyamine depletion (156).

Attempts to modulate polyamine concentrations often result in increased polyamine flux due to compensatory overexpression of polyamine-catabolizing enzymes (157,158). Accelerated flux leads to the depletion of acetyl-CoA and ATP and the generation of harmful compounds such as H_2O_2 that may lead to tissue damage and inflammation, and subsequently to tumor formation. Whether arginase-dependent production of ornithine is relevant for the increased polyamine metabolism in tumors has not been demonstrated to our knowledge.

Therapeutic arginine deprivation

New therapeutic approaches make use of the sensitivity of certain tumors to arginine deprivation by treating patients with PEGylated arginine deiminase (L-arginine iminohydrolase), a stabilized form of the enzyme that rapidly reduces plasma arginine concentrations by hydrolyzing arginine to citrulline. The therapy was well tolerated in patients with unresectable hepatocellular carcinoma, even when plasma arginine levels were below the detection limit for 3 months (159). Another approach is the combination of arginine deprivation followed by canavanine treatment (160), an arginine analogue that is incorporated into proteins and causes misfolding. Cancer cells *in vitro* were more susceptible than healthy cells.

Effects of arginine availability on TH1 and T_H2 responses

Schistosomiasis

Schistosomiasis is a chronic helminthic infection that causes a T_H2-type inflammation in response to the eggs deposited in the intestines and liver, and results in hepatic fibrosis and portal hypertension. T_H2-type cytokines, such as IL4 and IL13, induce alternative activation of macrophages, including arginase 1 expression.

As mentioned above, arginase 1 ablation in hematopoietic cells aggravates schistosoma pathology (92) and comparable results were found for Mesocestoides corti infections in IL4-deficient mice, where arginase expression in macrophages is diminished (161). The decreased TGFβ and increased IL12/IL23p40 production of peritoneal exudate cells in mice with arginase 1-deficient macrophages appear to account for the observed excessive pathology, as blocking IL12/IL23p40 production ameliorated the disease-associated pathology considerably and improved survival (162). Collectively, the data therefore support the hypothesis that the immunoregulatory function, i.e. suppression of T_H17 and T_H1 responses, is the main function of arginase 1 in macrophages.

Interestingly, a greatly enhanced fibrosis in lung and liver, portal hypertension, increased granuloma size, a significant increase in mast cells and eosinophils, and an unchanged schistosoma burden were found in *Cat2*$^{-/-}$ mice (58). Despite the increased pathological response, inflammatory cytokine production of both T_H1 cytokines (IFNγ) and T_H2 cytokines (IL4, IL13) was reduced in these mice. Since both macrophages and granuloma-derived fibroblasts of *Cat2*$^{-/-}$ mice show elevated arginase activity, the authors attribute the reduced T-cell proliferation to local arginine deprivation and suggest that CAT2 both supports NO production and suppresses arginase activity. One possible mechanism that might explain this observation is the reduced production of N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) in *Cat2*$^{-/-}$ deficient mice. Macrophages that can no longer sustain high NO production by NOS-2 also produce less nor-NOHA, an efficient suppressor of arginase activity. In any case, the data on arginase 1- and CAT2B-deficient mice strongly support the notion that arginase 1 overexpression inhibits T-cell proliferation and does not provide substrates for proline and polyamines production.

Asthma

A pronounced increase of arginase 1 expression in asthma has been demonstrated in both mice (163) and men (57), and a link with disease severity has been suggested. The precise location of the expressed arginase has however not yet been demonstrated convincingly. Weak arginase 1 protein expression was shown in bronchiolar-epithelium of healthy rats (164) and mock-immunized BALB/c mice. In OVA-sensitized and -challenged mice a modest increase of this expression was observed (163). In our murine model and in another study with experimental asthma in mice (57), however, arginase 1-staining by immunohistochemical techniques was only detected in macrophages of asthmatic animals and never in their bronchial epithelium. Animals, which were not sensitized to OVA, did not show any arginase 1-positive macrophages in their lungs as tested by immunohistochemistry on lung tissue or Western blotting of lung protein extracts (165). Ablation of *Arg1* in macrophages (*Arg1*$^{fl/fl}$/*Tie2Cre*$^{tg/-}$) led to an improvement of peripheral lung function in OVA -treated mice, without altering air hyperreactivity and lung inflammation (165).
In another series of experiments with arginine- rather than *Arg1*-deficient mice (manuscript in preparation: (166)), we found that circulating arginine concentrations primarily affected airway resistance in the larger airways. We, therefore, hypothesized

that ablation of *Arg1* in lung macrophages increases the parenchymal arginine concentration, which, in turn, facilitates NO production and smooth-muscle relaxation in the peripheral airways, but not the larger conducting airways.

Conclusion

Macrophage arginase 1 can in cooperation with cationic amino acid transporters control ambient arginine availability by efficiently depleting arginine locally. In infectious diseases, this suppresses not only proliferation of inflammatory T-cells, but also decreases collagen deposition by fibroblasts. The often cited causal link between arginase activity and increased collagen production does not seem to play a role in wound healing or infectious diseases characterized by excess fibrosis. The same holds true for the claim that increased arginase activity is sufficient for increased polyamine production in disease. Alternatively activated macrophages rather limit both processes by controlling local arginine concentrations.

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SYNOPSIS AND SCOPE OF THE THESIS

SYNOPSIS AND SCOPE OF THE THESIS

L-arginine is a basic amino acid in physiological fluids. Arginine is a precursor for proteins, for creatine and for the signaling molecules agmatine and nitric oxide. Arginine is also a secretagogue for growth hormone, insulin, glucagon and prolactin. Its content is relatively high in seafood, watermelon juice; nuts, seeds, algae, meats, rice protein concentrate, and soy protein isolate (1,2) but low in the milk of most mammals (including cows, humans, and pigs)(3,4). In addition, preterm infants who represent 10-12% of newborns, exhibit arginine deficiency (5), resulting in hyperammonemia and multiorgan dysfunction (6). It has also been shown that arginine supplied in current diets is inadequate for maximal growth of milk-fed piglets (7) or maximal reproductive performance of swine (8). Thus, arginine nutrition remains a significant concern in both human and animal health. This thesis aims at characterizing the role of arginine in growth and chronic inflammatory diseases through manipulation of local or systemic arginine concentration using conditional knockout and other transgenic mouse models.

Macrophages are phagocytic cells involved in daily garbage clearance on the one hand and play an important role in innate and adaptive immunity on the other hand. Immunologically active macrophages are a heterogeneous cell population with two outer extremes, classified as M1 and M2 macrophages, or classically and alternatively activated macrophages (9), respectively. M1 macrophages (also described as inflammatory macrophages) use arginine for the generation of nitric oxide by inducible nitric oxide synthase (NOS2), whereas the immunosuppressive M2 macrophages deplete arginine through catabolism by arginases (ARG).

This raises the interesting question whether and to which extent arginine metabolism determines macrophage function. Arginine metabolism is not a simple marker for the activation status of macrophages, but also has a strong effect on the outcome of many diseases. The **Chapter I** of this thesis reviews the arginine metabolism of macrophage and its role in chronic diseases.

To further study the role of arginine – several knockout (KO) models were generated that allowed us to investigate arginine metabolism or to manipulate arginine availability. The models that were used affect arginine synthesis (conditional KO of arginine-synthetase, *Ass*), transport (conditional KO of cationic amino acid transporter 1, *Cat1*) or degradation (conditional KO of arginase 1, *Arg1*) or resulted in excessive arginine degradation (intestinal overexpression of rat arginase 1, F/A2). **Chapter II** of this thesis describes the chosen strategy for the generation of conditional knockout mice, and its advantages and limitations. The development of optimal screening and selection protocols for embryonic stem cells after transfection is described. We demonstrate that a PCR-based approach to amplify the homologous arms of a targeting construct is not very efficient due to the error rate of the heat-stable DNA polymerase. This chapter is a trouble-shooting guide for the generation of a conditional KO mouse.

To elucidate the role of arginase 1 in chronic diseases, a tissue-specific KO mouse of arginase 1 was created using Cre/loxP recombination. $Arg1^{fl/fl}$ mice were crossed with *Tie2Cre* or *LysMCre*

mice to eliminate ARG1 expression from all hematopoietic and endothelial cells or from granulocytes and macrophages, respectively. **Chapter III** shows that ablation of *Arg1* in hematopoietic and endothelial cells did not affect OVA sensitization and flow resistance but attenuated tissue elastance (H) and tissue resistance (G) in a murine model of asthma, indicating that *Arg1* ablation mainly affected the lung parenchyma (10), but not the airways.

It is also possible that the main function of macrophages (and possibly other arginase-expressing cells) is the control of arginine availability. Interestingly, there is a limited period of perinatal intestinal arginine synthesis in rodents and pigs – which is later abolished by arginase expression. Mammalian milk contains too little arginine for normal growth, but its precursors proline and glutamine are abundant; but the small intestine of rodents and piglets produces arginine from proline during the suckling period. Parenterally fed, premature human neonates frequently suffer from hypoargininemia. These findings raised the question whether the human small intestine also expresses the enzymes that enable the synthesis of arginine from proline and/or glutamine**. Chapter IV** is a histological study which shows that the human intestine indeed has the capacity to synthesize arginine, and that this capacity is strongest in the first three years after birth. However, the function of the arginine thus produced is not clear (11).

It is believed that perinatal intestinal arginine synthesis compensates for insufficient arginine concentrations in maternal milk to ensure appropriate development. To test this hypothesis, we inactivated arginine synthesis in the gut by specifically inactivating ASS expression in the enterocytes of the small intestine. In contrast to mice overexpressing arginase 1 in the enterocytes (F/A2 mice), which showed severe retardation in general growth, reduced hair growth and impaired B cell maturation, mice deficient for ASS in their enterocytes showed no major phenotype. In **Chapter V** we characterized the mice with intestinal ASS deficiency. Absence of intestinal arginine biosynthesis in ASS deficient mice resulted in an increased production of citrulline by the gut and an increased hepatic absorption of arginine. Furthermore, arginine synthesis in the kidneys was increased (12). The question remained what might be the reason for the dramatic phenotype of F/A2 mice (clearly not the lack of intestinal arginine synthesis, but rather the excessive breakdown of not only intestinal arginine but also arginine from the diet) – in other words, what are the mechanisms underlying these dramatic effects of arginine deficiency?

The phenotype of F/A2 mice is reminiscent of the phenotype of IGF-1 deficient mice, which are also growth retarded. We hypothesized that the syndrome results from an interaction of the growth-hormone/IGF-1 axis and the amino acid-sensing GCN2 stress-kinase pathway. In **Chapter VI** we characterized this mouse model *in vivo* in combination with several *in vitro* cell culture models. We show that arginine deficiency inhibits the release of growth hormone from the pituitary gland and ubiquitously activates the stress kinase GCN2 leading to a general shut-down of protein synthesis. Our findings demonstrate the signaling properties of arginine on the growth hormone axis and on the protein synthesizing machinery (13).

In the general discussion, we review known or claimed beneficial effects of arginine supplementation and analyze the available evidence from our own studies and those of others.

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CHAPTER II

EXPERIENCES WITH THE CONSTRUCTION OF CONDITIONAL KNOCKOUT MICE

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CHAPTER II

Introduction

The development of techniques that allow the modification of the DNA sequence of an allele of a gene in embryonic stem cells enables us, in combination with microinjection techniques, to deliberately modify the expression of genes in a living organism, usually the mouse. The deletion (knockout) or replacement (knock-in) of specific genes has proven to be a powerful tool. In this chapter, we comment on our experience with the generation of conditional knockout mice of the genes that code for argininosuccinate synthetase (*Ass*; VM), arginase 1 (*Arg1*; SS) and cationic amino-acid transporter 1 (*Cat1*; CCdT).

Conventional knockout mice (deletion of a specific gene in every cell of the organism) are often not viable. This problem can be overcome by making "conditional" knockout mice that allow the deletion of a gene in a specific tissue or at a specific developmental stage. Deletion in specific tissues or at a specific time point can be achieved with just one genetic modification of the gene of interest, that is, the insertion of two recognition sites for an enzyme with recombinase activity. Mice harboring such a modified allele are crossed with mice expressing the recombinase under control of a promoter that determines the spatiotemporal expression of the recombinase and thereby the spatiotemporal deletion of the DNA fragment surrounded by the recombinase recognition sites. Two systems are most widely used and discussed in this chapter, viz, the cre/loxP system and the *flp*/frt systems. LoxP and frt refer to the recombinase recognition sites, and CRE and FLP to the respective recombinases (1-4). There are now more than 100 cre mice available (see http://www.mshri.on.ca/nagy/), permitting deletion of genes in specific cell types (macrophages, B-cells, enterocytes, hepatocytes, endothelial cells to name a few) or at specific times using a recombinase that is modified to contain the ligand-binding domain of a member of the steroid-hormone receptor family. Most often, this ligand-binding domain is modified itself to respond only to a ligand that is not endogenously produced. In practice, the combination of a modification of the estrogen-receptor ligand-binding domain and the ligand tamoxifen is most often used.

The first step in the generation of a conditional knockout mouse is the design and construction of an appropriate vector that is then introduced into embryonic stem cells by transfection. This paper describes a PCR-based method for vector construction and specifies screening protocols for the embryonic stem cells after transfection. The approach is summarized in Figure 1.

The Targeting Plasmid

A targeting plasmid consists of two regions with target homology (hereafter called flanking regions; Figure 2), the region of the gene to be deleted, and genes that are used as selection tool. The flanking regions are placed at either end of the sequence to be deleted and are identical to the

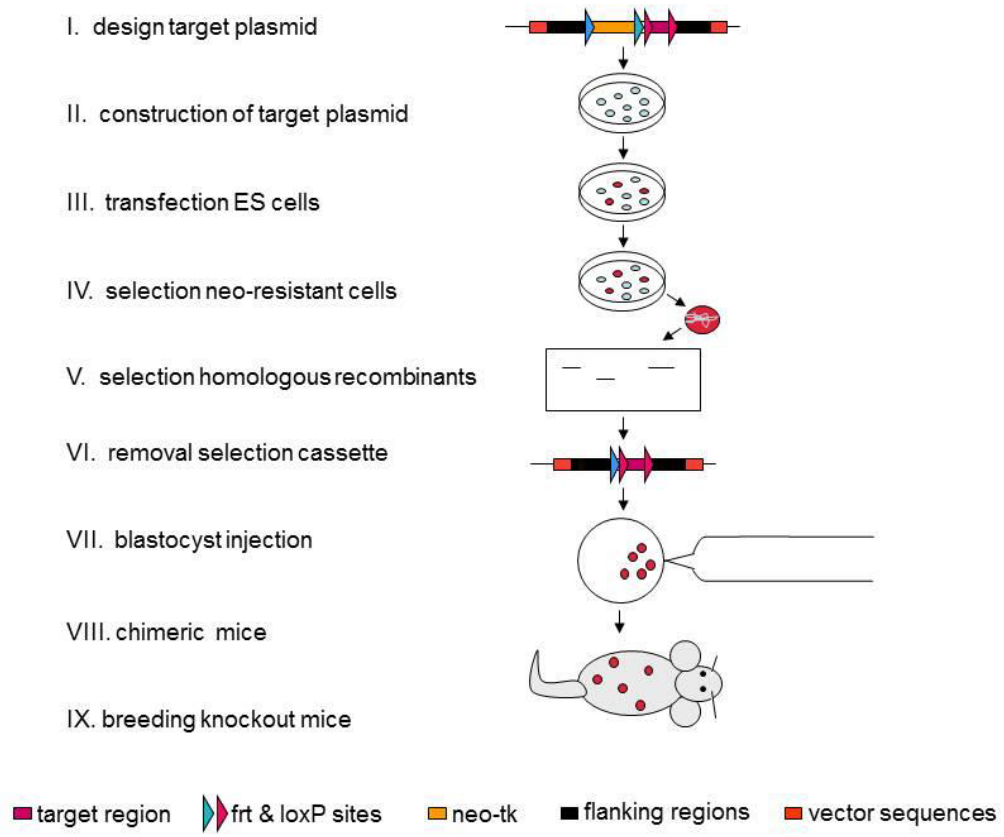

Figure 1: Overview of the design and construction of the targeting plasmid, the selection and characterization of the targeted ES cells, and the procedure to modify the germline of mice.

sequences surrounding the target sequence. The flanking regions are required for homologous recombination, that is, for the site-specific insertion of the construct into genomic DNA. In our strategy, the exon(s) or gene to be deleted are flanked by loxP sites, whereas the selection tools (neomycin phosphotransferase ("neo") and viral thymidine kinase ("tk")) are surrounded by frt sites.

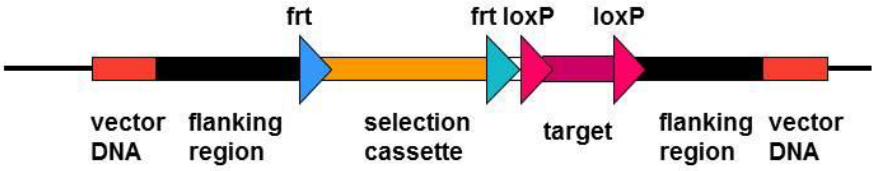

Figure 2: Structure of a targeting construct. The fragment to be deleted is surrounded by "loxP" sequences ("floxed"). Adjacent to the floxed sequence, a selection cassette is inserted, which itself is surrounded by "frt" sequences for later removal. The target sequence and the selection cassette are located in between the "flanking regions", which are identical to the sequences surrounding the part of the gene that is to be deleted. At both ends of the construct, fragments of the vector can be left behind to facilitate selection. See text for details.

Vector construction has been greatly facilitated by the availability of the complete mouse genome (http://www.ncbi.nlm.nih.gov, http://www.onderzoekinformatie.nl) and additional information on SNPs and strain differences. This made it possible to use a PCR-based approach for vector construction, which also facilitates the introduction of additional restriction sites necessary for later cloning steps. The advantages and pitfalls of this approach will be discussed.

Selection of target region to be excised

One can delete one or more crucial exons or the entire coding sequence to inactivate the gene of interest. Deletion of one or a few exons is often preferable, since the efficiency of the CRE recombinase seems to be at least partially dependent on the distance between the loxP sites (5).
The region chosen for deletion should either code for domains that are important for the function of the gene product (eg. the active site of an enzyme) or should result in a frame shift with premature stop codons. One should nevertheless be aware of alternative splicing which could result in a partially functional gene product excision (e.g. (6)). Literature data on lethal mutations in man and mouse should be consulted, because they can be very valuable for the development of the knockout strategy. Examples are: natural mutants (in the case of the arginosuccinate synthetase gene, we selected exon 13 for deletion, because the deletion results in a frame shift and natural mutations in this exon led to undetectable levels of enzyme activity (7) or results known from conventional knockout mice (in the case of the arginase-1 gene, we selected exon 4 for deletion, because the conventional arginase I knockout mouse was made by deleting this exon and died between 10-14 days after birth (8)).

Prerequisites for homologous recombination: length of the homologous regions

Between 2 and 10 kb, the relationship between the length of the homologous region and the frequency of targeting events is exponential and shows saturation at 14 kb of homology (9). Therefore, a minimum length of 2 kb is recommended for the flanking region to support optimal homologous recombination. However, 1.5 kb of homologous region at one end and 5 kb at the other end proved sufficient for efficient recombination of the Glutamine Synthetase gene (Y. HE, AMC Liver Center, Amsterdam). In our case, we used approximately 4.5 kb of homologous region on either side of the construct. Although this was thought to be advantageous for homologous recombination, long flanking regions as e.g. found in pROSA26 (10) make screening of clones by Southern or PCR more difficult. For this reason, many constructs contain a long and a short flanking region.

Comment

An important parameter that is often neglected when selecting a target region for vector construction is the presence of repeats in the sequence to be amplified. Such repeats should be avoided since they are troublesome during amplification by PCR.

Recombinase systems

Cre-loxP system

The P1 bacteriophage cyclization recombination (*cre*) recombinase recognizes a pair of *loxP* sites flanking a DNA sequence. *LoxP* sites are 34 bp long and composed of an 8bp core, which determines directionality, flanked by 13 bp of palindromic sequence on either side (Figure 3A). The natural occurrence of this exact sequence is unlikely in any eukaryotic genome. Sequences flanked by *loxP* sites are said to be "floxed". The *cre* enzyme brings the two *loxP* sites into juxtaposition. If the *loxP* sites flank a DNA segment in a cis arrangement and are oriented in the same direction, CRE recombinase mediates excision and circularization of the flanked segment (Figure 3B). If the *loxP* sites flank the DNA segment in a cis arrangement and are oriented in opposite directions, CRE recombinase mediates the inversion of the segment (Figure 3C). If the *loxP* sites are located on different strands of the DNA and are oriented in the same direction, CRE recombinase can mediate translocation of the segment (not shown).

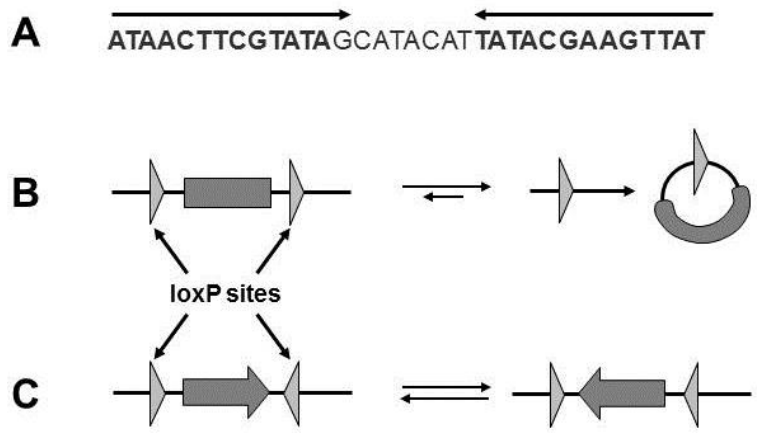

Figure 3: LoxP sites. Panel A: The nucleotide sequence of loxP sites. Note the inverted repeat surrounding the asymmetric core sequence. Due to their asymmetric core, the orientation and position of the loxP sites determines the outcome of the recombination event. In conditional knockout strategies, the aim is excision of the fragment which is achieved by placing 2 loxP sites on each side of the sequence of interest in the same orientation (B). With an opposite orientation of the loxP sites, an inversion of the floxed region is obtained in presence of CRE (C).

Flp-frt system

The yeast *flp* recombinase (4, 11) recognizes target sites called frt (12, 13). A full-length frt site is 48 bps long and, similar to a loxP site, consists of an asymmetric central 8bp spacer sequence, flanked by 13bp inverted repeats (Figure 4). However, in contrast to loxP sites, it contains a third 13bp direct repeat at one side (14). Because the Flp/frt system is derived from S. cerevisiae, its optimal working temperature is 25-30˚C. At 37˚C, the activity of the enzyme is dramatically reduced (15, 16), so that Flp is less suitable and less frequently used to engineer genome modifications in mammals. Attempts to overcome this limitation have led to the development of an "enhanced" mutant FLP (FLPe) by cycling mutagenesis. FLPe is said to have a better thermostability and activity (16, 17) and a more efficient function in mammalian cells (16, 18).

Figure 4: The nucleotide sequence of frt sites. Note inverted repeat surrounding asymmetric core sequence, which contains a convenient XbaI restriction site. Also note that the left-sided repeat needs to be present as 2 copies for higher efficiency.

Selection tools

A homologous recombination is a very rare event compared to random integration. Based on our experience, homologous recombination occurs in ~50% (ROSA locus; M. Konings) to 0.05% of all integration events (arginase I locus). Selection is therefore necessary to identify the clones in which homologous recombination has occurred and to distinguish these clones from the clones in which the modified sequence is randomly integrated into the genomic DNA. To achieve this, a positive-negative selection protocol is commonly used (19). The positive selection tool is used to identify ES cells with the targeting sequence integrated into their DNA. Neomycin phosphotransferase (neo) is often used as a positive selection marker to select for ES clones in which the construct is integrated. Only clones that have integrated the construct will be able to grow in G418-containing medium as neomycin phosphotransferase will detoxify the compound.

The negative marker can be used to select for homologous recombination of the targeting construct (Figure 5A) or to select for successful removal of the positive selection marker in the ES cells prior to blastocyst injection (Figure 5B). As a tool to select for homologous recombination, the negative selection marker has to be placed at the outer end of one of the flanking regions, whereas the positive selection marker is placed in the middle of the construct. Positioning the negative selection marker at one extremity of the targeting construct (Figure 5A) will lead to elimination of this marker during homologous recombination. Addition of the selection compound to the medium will then kill all cells that still express the negative selection marker due to random integration, whereas clones with homologous recombination will not be affected. Viral thymidine kinase (tk) is a commonly used negative selection marker, as it confers

sensitivity to ganciclovir in the ES cells. Phosphorylated ganciclovir is a toxic substance, so that cells can only grow if thymidine kinase is not or no longer present.

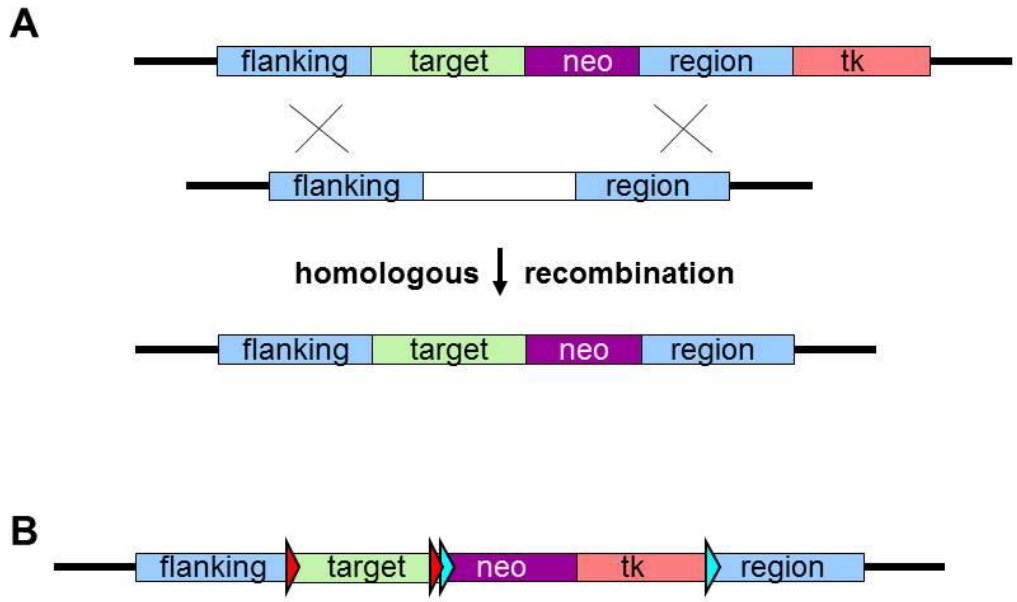

Figure 5: The two applications of a negative selection marker. In panel A, a negative selection marker is placed at either end of the construct to select against random integration. In panel B, the negative marker is placed in the middle of the construct and surrounded by recombinase recognition sites for removal after recombination.

Removal of selection markers

To select for successful removal of the positive selection marker, both positive and negative selection tools have to be present in between the two flanking regions (Figure 2). There are 2 strategies to remove the selection markers. One approach uses three loxP sites that surround the target sequence and the selection marker. In the other approach, the target sequence and the selection markers are surrounded by loxP and frt sites, respectively. After selection for the positive selection marker and identification of a correct clone by the appropriate screening protocols, the selection tools are removed by transient transfection with either a *cre*- or *flpe*-expressing plasmid, depending on whether the selection tools are surrounded by loxP (Figure 6) or frt sites (Figure 2). *In vivo*, loxP sites are preferable over frt sites as recognition sites for a recombinase, because CRE is a more efficient recombinase than FLP and because there are many more mouse strains harboring tissue-specific expression of *cre* than *flp*. Therefore, the gene fragment to be excised *in vivo* is usually surrounded by loxP sites.

Figure 6 shows the use of 3 loxP sites for gene targeting. Transient transfection of ES cells with a *cre*-expressing plasmid will ideally yield three products: cell clones, in which only the selection tool is deleted, cell clones in which only the target sequence is deleted, and cell clones with excision of both the selection tools and the target sequence. The first type of clones is the desired

outcome, whereas the second and third type of clones will generate conventional knockouts. It is unfortunately not yet possible to predict if the desired type of clones can be obtained. We, therefore, chose for an approach in which the targeted gene fragment is placed between loxP sites and both positive and negative selection tools are present in the middle of the construct in between frt sites (Figure 2). This allowed us to first select for integration of our constructs into the genomic DNA and then to select for deletion of the positive-negative selection tool before the injection of ES cells into blastocysts.

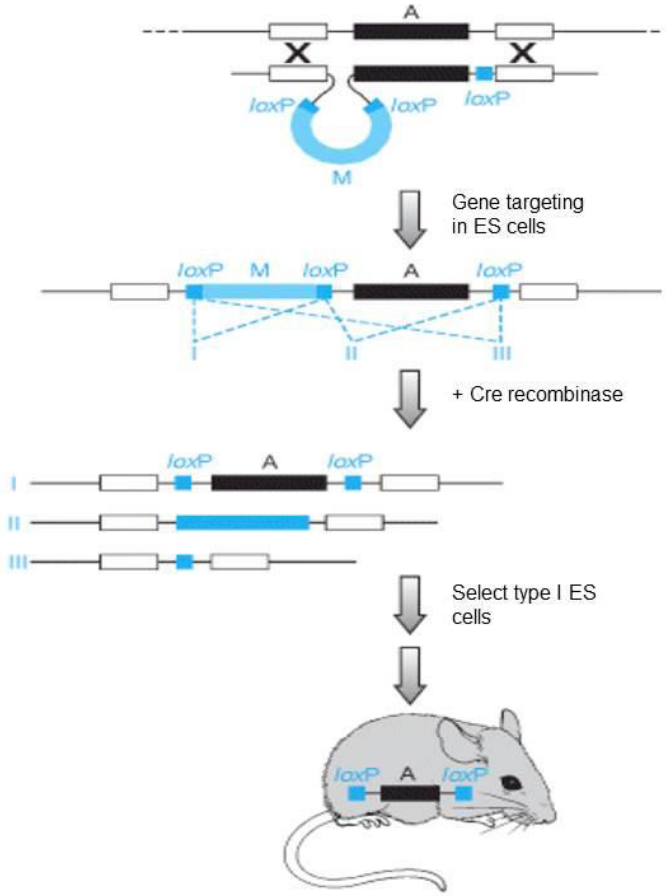

Figure 6: Gene targeting using loxP sites. Gene targeting using solely loxP sites can be used to inactivate a specific gene in a desired cell type. In the standard homolous recombination protocol, three loxP sites are introduced along with a selection tool "M" at the target locus "A". Subsequent transient transfection of a *cre* recombinase-expression plasmid or breeding a chimeric mouse with a deleter-*Cre* mouse results in recombination between the three loxP sites. In the desired Type I recombination, only the selection tool is removed. Both other recombinations generate convential unconditional knockouts.

Comments

1. An alternative to "select" against random integration by including a negative selection marker at the outer end of the targeting construct is the use of a high throughput screening protocol, e.g. a PCR-based screening for the presence of remaining vector sequence. This strategy will be discussed in section III A.

2. Multiple copies of loxP can lead to chromosomal instability during expression of *cre* (20).
3. An alternative to plasmid transient transfection with *cre* or *flp* to remove the selection tools is to cross mice carrying the properly recombined targeting construct that still contains the selection marker with a mouse expressing *cre* or *flp* in early development, i.e., before the germline is set apart. An example of a *cre*-expressing mice is the II2a-*Cre* mouse (21), while the "flipper" mouse is an example of an often used *flpe*-expressing mouse (22). The availability of the efficient flipper mouse (~50% frt excision (22)) has made the use of a negative selection marker like thymidine kinase obsolete, because the positive selection marker can now be removed *in vivo*. The use of solely the *neo* selection tool and its removal *in vivo* has 3 additional advantages:
   - one round of *in vitro* selection can be avoided (see section IV B), which decreases the chance that pluripotency of the ES cell is lost;
   - crossing the mice with a *flpe*-deleter mouse on a pure background spares one round of breeding towards a new background;
   - a targeted allele containing *neo* is available. Such alleles are often cripple (19, 23-26), allowing one to obtain not only animals with 0, 50% and 100% gene expression of the gene of interest, but also animals with intermediate expression levels (0-50% and 50-100%).

Cloning the target and flanking regions

PCR

Homologous recombination requires a 1.5 kb - 5 kb stretch of DNA on either side of the construct that is identical to the sequence to the wild type DNA (27). The efficiency of homologous recombination depends on the degree of sequence similarity. When the construct is generated by PCR, the use of a high fidelity of the DNA polymerase for the amplification of the flanking regions from genomic DNA is therefore a prerequisite.

The different commercially available thermostable DNA polymerases show a ~20-fold difference in error rates (28, 29). The DNA polymerases from thermophilic bacteria (e.g. Thermus aquaticus) miss proof-reading 3'-5'- exonuclease activity and lack the ability to synthesize products larger than 5 Kb. DNA polymerases from hyperthermophilic archaea (e.g. Thermococcus fumiculans ("Tfu") and litoralis ("Vent") and especially Pyrococcus furiosus ("Pfu") and abyssi) have low error rates, but the stronger the proofreading activity, the lower the product yield of long DNA fragments. Therefore, blends of the non-proofreading Taq and a proof-reading enzyme are often used: "LA" (long and accurate (30)) PCR. According to the manufacturer, the Accutaq LA DNA Polymerase (Sigma), which we used for amplifying the homologous arms, has a 6.5-fold greater fidelity than Taq polymerase. However, in 3 different targetings, we observed 27 differences with published sequences in 6.9 kb of DNA (0.4%). Some of these differences may reflect strain differences (published sequences are often C57BL/6 or

BALB/c, whereas the ES cell line came from 129P2/OlaHsd mice). In the case of arginase I construct, we found 5 mismatches in the exonic sequences, which did not lead to a change in amino-acid coding. We did not correct sequences, if they did not affect a consensus splice site or a coding sequence. However, when a coding sequence was affected (e.g. (GCG (Ala) to GTG (Val) in exon 6 of the arginase-1 gene), we did correct the mismatch.

Another problem of the PCR approach that we encountered was the presence of highly repetitive sequences in the region to be amplified. In the 5'-flanking region of the ASS targeting construct, for instance, a 36 bp TA repeat was present in the middle of the 4.2 kb fragment. To amplify this sequence, we tried several high-fidelity Taq enzymes (Pfu "Ultra High" Fidelity and "Herculase" DNA Polymerases (Stratagene); AccuTaq Polymerase (Sigma)), but none of them was able to amplify the sequence. Eventually, we had to break up this flanking region into 4 fragments of approx. 1 kb each.

Comments

Our choice to amplify the flanking regions using genomic rather than plasmid- (BAC-) derived DNA was based on the limited availability of BAC clones when we designed our approach. However, since BAC-derived DNA is ~30,000-fold purified per weight unit for a particular 100 kb fragment relative to primary genomic DNA, the number of PCR cycles necessary to amplifly the DNA is reduced by ~17, which strongly reduces the fidelity issue.

Electroporation of the targeting construct

Purification of the targeting construct

Prior to electroporation in the ES cells, the construct is either linearized by restriction enzymes cutting in the vector backbone or digested with two restriction enzymes to remove the vector backbone. Complete removal of the backbone of the vector has the advantage of working with a "clean" construct without additional sequences that might interfere with homologous recombination in the cell. On the other hand, linearization avoids extra steps of purification of the targeting construct. Furthermore, the additional vector sequences can be used for a PCR screening strategy to eliminate clones with random integration quickly. This approach is described in section *III.a (Elimination of randomly integrated clones)*.

There are several methods to purify large DNA fragments by electrophoresis (*purification with commercial kits, electrophoresis in low melting point agarose followed by acid-phenol extraction* or *electro-elution)*. We used electroelution, using a bridging system to concentrate the DNA on a dialysis membrane (Figure 7A). The purified DNA was then precipitated in ethanol for electroporation.

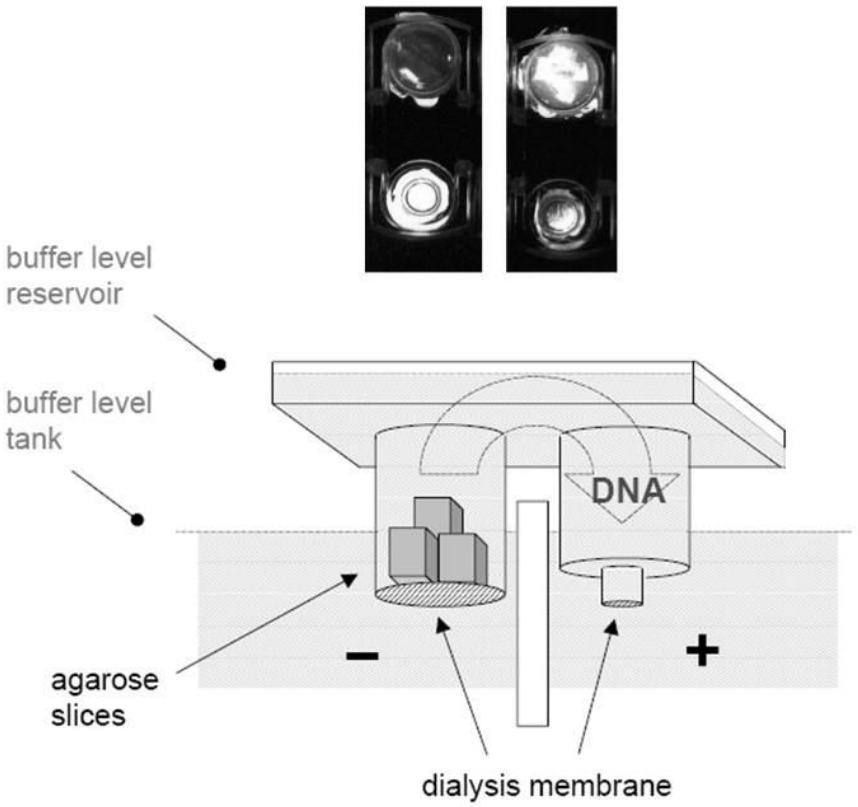

Figure 7: Electroelution. Panel A: Schematic representation of the electro-elution system used to purify the targeting vector prior to electroporation. The two cylindrical reservoirs communicate through a buffer compartment on top. DNA migrates in an electric field (open arrow) from the agarose slices (in large reservoir, left) onto the dialysis membrane (in small reservoir, right). Panel B: after electroelution, the DNA concentrates on the dialysis membrane. The left picture shows an efficient transfer of DNA to the dialysis membrane, whereas the right picture shows an inefficient transfer (a high proportion of DNA remained in the agarose).

Comments

1. DNA that is to be used to generate transgenic animals should be protected as much as possible from UV light (i.e. day light, UV light box). Long-wave UV light or a Dark Reader® using visible light (Clare chemicals), is recommended to visualize DNA. We are aware of a case in which undue exposure to UV light caused mutations in the majority of otherwise successfully targeted ES cells.
2. Residual ethidium bromide will interfere the efficiency of homologous recombination. Traces of ethidium bromide in a DNA preparation can be easily detected on an agarose gel from which ethidium bromide has been omitted. If DNA is detectable under UV light, the preparation should be re-extracted. For the same reason, working surfaces and equipment (UV tray; scalpels) have to be clean, when isolating fragments from agarose gels.

3. During electroelution of DNA, the agarose blocks should be stacked properly to ensure efficient transfer (Figure 7B, left picture). Floating pieces of agarose result in inefficient transfer of DNA (Figure 7B, right picture) and are a major source of DNA loss.

Choice of ES cell line

The choice of ES cell line is an important factor to achieve high success rates. The 129P2/OlaHsd-derived IB10 ES cells (a subclone from (male) E14 ES cells (31)) was used successfully by our group for the generation of 4 conditional knockout and 3 knock-in mice.

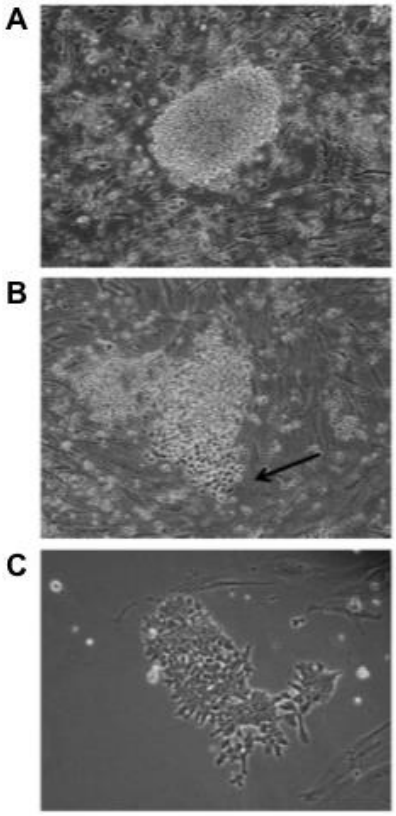

***Figure 8: The phenotype of ES cells cultured* in vitro.** Picture A: a colony of undifferentiated ES cells cultured on mouse embryonic fibroblasts (MEFs). The cells are spherical and show no boundary at the circumference of the colony. Picture B: a colony of differentiated ES cell with part of the cells exhibiting a high degree of differentiation (arrow). The cells are no longer spherical. Picture C: a colony of ES cells grown in BRL-conditioned medium. Although still undifferentiated, this ES colony is very different in appearance from one grown on MEFs.

Electroporation of IB10 ES cells

ES cells from an 80% confluent 25cm^2 culture flask were electroporated with 25 µg DNA in a final volume of 200 µL sterile PBS. At a setting of the capacitance at 1 µF, the time constant should not exceed 0.1. After electroporation, the ES cells were seeded in two 10 cm dishes, containing medium without selection compound.

The exposure of the electroporated ES cells to the selection medium (200 µg/mL G418) was started 24 hours after electroporation. Five days later, resistant colonies become visible, with individual colonies becoming visible after 6 to 8 days. The colonies should not be left to grow too big, since this induces differentiation of the ES cells. Figure 8A shows an undifferentiated ES colony with cells retaining their spherical shape. On the other hand, when the ES cells become differentiated, they acquire a rectangular shape (Figure 8B) and should be disgarded. Series of 16 undifferentiated colonies were picked and transferred to 96-well plates containing neomycin-resistant mouse embryonic fibroblasts (neo-MEF). After 2-3 days, when the cultures are around 60% confluent, they are split onto two plates, one containing neo-MEFs and the other 150 µL of BRL (Buffalo Rat Liver) conditioned medium. The cultures on neo-MEFs were grown to semiconfluence (2-3 days) and frozen. The colonies on BRL medium were expanded for isolation of genomic DNA. Note that these ES cells look different from MEF-supported ES cells (Figure 8C).

Comment

Recombinant ES cells should be grown under selection pressure until the selection tool has been excised to suppress the growth of wild-type cells. The requirement for a "deep split" (see section IV.a.) shows that selection pressure should be a standard part of the procedure.

Screening for Homologously Recombined Clones

Once neomycin-resistant clones originating from single cells have been picked, they need to be screened for proper integration of the construct into the targeted gene. The picked clones are seeded individually in 96-well plates. It was common to observe differences in the growth rate among the recombinant cells. Therefore, care was taken not to allow cells to grow too confluent, thereby avoiding differentiation. Because screening is a tedious and time-consuming procedure, it is recommended to temporarily freeze an aliquot of promising ES colonies, while going through the selection with the remaining portion of the clone. Two ways of freezing down the cells were used. A computer-assisted cooling device, in which the cryotubes were placed, in a temperature-controlled chamber with a reduction in temperature of 1ºC per minute to -80ºC, before transferring the tubes to liquid nitrogen. When this machine was not available, we cooled the cryotubes in a foam box containing liquid isopropanol based on the same principle as the Mr Frosty labware (Nalgene) in a -80ºC freezer before transferring them to liquid nitrogen.

Elimination of randomly integrated clones

After electropration of the targeting vector, the vector either integrates randomly in the genomic DNA (RI) or recombines homologously with the gene of interest (HR). An interesting difference between these two ways of insertion is that the extremities of the vector are kept during randing integration, but lost during homologous recombination (Figure 9A). We used this difference for a preliminary test to remove clones with randomly integrated DNA. We left the multiple cloning region (MCR) on either end of the linearized fragment or only linearized targeting construct. A MCR- or vector-specific primer and a construct-specific primer were used. Since the outer ends of the construct are lost during homologous recombination, only randomly integrated clones still have the MCR- or vector-specific sequence necessary for primer binding (Figure 9B). This "short" PCR approach worked well for some targeting vectors, e.g. ASS, that is, ~70% of the clones that were randomly integrated could be eliminated. We therefore suggest linearizing the targeting construct near the shortest flanking sequence. When the construct was restricted at the outer side of the MCRs before electroporation, leaving no backbone, this short PCR did not work efficiently. Probably, the extremities of the construct are nibbled off by nuclease activity before integration. Clones yielding a PCR product were discarded; clones negative for this PCR were treated as potential candidates for proper homologous integration and investigated further.

Selection of homologously recombined clones

Long PCR

Clones negative for the "short PCR" were subjected to a "long PCR". To discriminate the recombined allele from the wild-type allele, the primers for the long PCR were chosen to be specific for the recombined allele. The target-specific primer sequences were chosen in the selection markers, the lox P sites, or the frt sites in the center of the construct and in the genomic sequence outside that present in the targeting vector sequence (Figure 9C). This PCR yields a specific PCR product with a known size, when homologous recombination has taken place. The PCR product is then restricted with appropriate enzymes and sequenced to confirm the exact insertion of the targeting vector. The long PCR yields very detailed information on the integration of the DNA. We, therefore, recommend to perform this long PCR on both sides of the integrated vector, if this is possible. Finally, all crucial sequences are sequenced to check for any mutation.

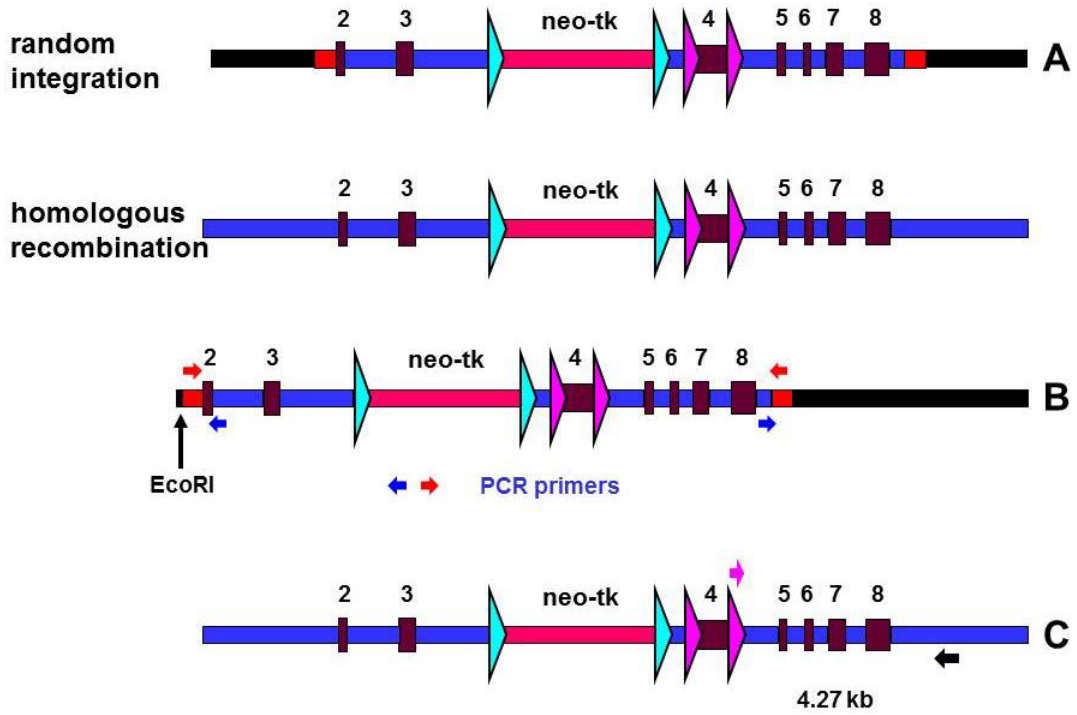

Figure 9: PCR-Screening for homologous recombination. Panel A: Configuration of a targeting construct after random integration or after homologous recombination. The vector-specific sequences at the ends of the targeting construct (black) remain only present if the construct is randomly integrated. Panel B: This structural feature allows a negative selection for properly integrated clones by "short" PCR. Primers are designed specifically for these sequences, which will only give a band for randomly integrated clones. It is obvious that PCR at the right side (long vector sequence left) is less sensitive to "nuclease nibbling" than PCR at the left side. Panel C: Screening for homologous recombination by "long" PCR. By using primers in the endogenous DNA outside the targeting construct and in the selection marker or recombination sites, a PCR product that is unique for the recombined allele is produced. This PCR product can then be used to sequence crucial parts of the modified allele without interference from the wild-type allele.

Southern blot

Clones positive for the long PCR on both sides were further tested by Southern blot to check for clonality, that is, checked for the presence of an equal number of copies of the recombined and the unmodified endogenous allele to test for the presence of wild-type ES cells that were not removed during the positive selection procedure. This Southern-blot does also reveal the presence or absence of additional random integrations.

The DNA sequence used to probe such a Southern blot should be localized external to the flanking regions of the construct. The quality of the probe determines the quality of the result. We found that a relatively short probe that is specific and contains no repetitive elements is more efficient than longer probes containing such repetitive elements. Also, it should be close to the boundaries of the construct to avoid excessively long sequences. Exons are preferable to introns because introns more often contain repetitive sequences and, therefore, bind non-specifically to sequences elsewhere, resulting in smears as depicted in Figure 10, left panel. However, sometimes the next exon is so far away that intronic sequences have to be used as probe. Any candidate probe should be checked for its specificity by blasting for the occurrence of relatively short sequences (>12 bp) that are shared with other regions (comparison with the mouse genomic database) and then tested on a Southern. There is no golden rule to select a sequence for a Southern probe, in the sense that intronic probes can be as good as exonic probes as shown in Figure 10, right panel, where we used an intronic sequence of 300 bp for the Southern probe and obtained high specificity.

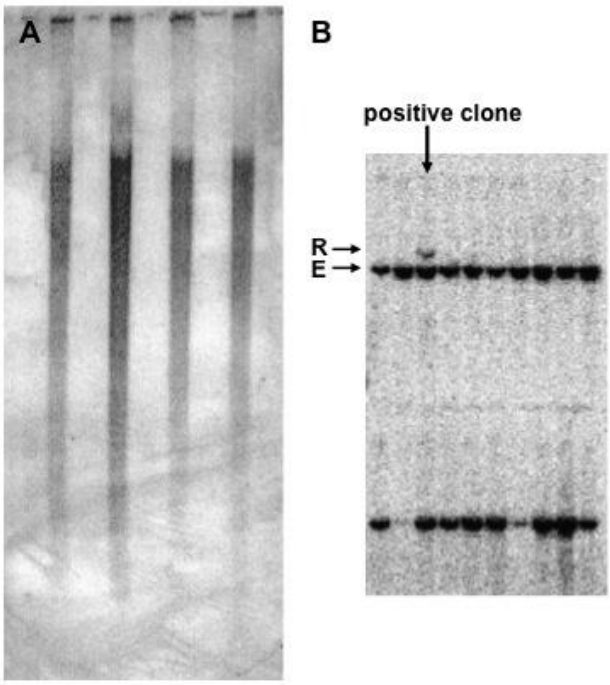

Figure 10: Southern blotting as tool to identify homologous recombination. Panel A: ES-cell DNA was digested with Xmn-I, blotted, and hybridized with an external intronic ASS probe (1.17kb). The probe generated the same staining pattern as ethidium bromide, demonstrating non-specific hybridization. Panel B: ES-cell DNA was digested with Blb-1, blotted, and hybridized with a short 300bp intronic arginase-1 probe. The wild-type allele is 6.1 kb long and the recombined allele 8.5 kb. Note the difference in intensity of the wild-type and recombined allele. The excess of wild-type DNA suggests that wild-type cells had escaped removal during neomycin selection.
R: Recombinant DNA fragment; E: Endogenous DNA fragment.

Comment

DNA isolated for genotyping may be difficult to dissolve. If the centrifugational force is limited, a more homogenous solution is usually obtained. Prolonged centrifugation during precipitation should, therefore, be avoided.

Removal of Selection Tools

Clonality of the recombined clone ("deep split")

The picked ES colonies are often contaminated with WT ES cells. These contaminant cells need to be removed before blastocyst injection. The clonality test, a Southern blot-based technique, allows us to visualize the intensity (that is, the prevalence in the population) of both the endogenous and recombinant alleles and to compare them. If the ES cells used on the blot originate from a pure recombinant clone, the intensities of the two bands should be equal. On the other hand, if the intensity of the endogenous band is stronger than the recombinant one, there is most likely a contamination with wild-type ES cells (Figure 10B). We observed contamination with wild-type ES cells in many cases as long as we stopped the G418 selection after picking the clones. It is therefore highly recommended to continue the drug selection throughout the whole procedure. To remove the remaining wild-type cells, a deep split has to be done and the resulting "single-cell" ES cultures have to be subjected to a new round of G418 selection.

Removal of the neo-tk cassette

The presence of a selection cassette can interfere with the expression of a nearby gene (19, 23-26). Furthermore, ES cells that express tk are less likely to be incorporated into the male germ line (23, 32). It is therefore recommended to remove the neo-tk cassette or any other selection cassette in the selected ES-cell clones prior to blastocyst injection. The excision of the cassette can be achieved by transient expression of either *flpe* or *cre* recombinase through electroporation of either a *flpe*- or *cre*-expressing plasmid. In our constructs, we use the Cre-LoxP system to achieve specific gene inactivation, so that the Flp-Frt system remained to remove our selection cassette.

Comments

1. The *flpe*-expression plasmid (Figure 11A) is a fastidious plasmid. After electroporation into e.g DH5-α bacteria according to the Biorad standard electroporation protocol, the incubation temperature of the bacteria should not exceed 30˚C. At 37˚C, the apparent size of the plasmid doubled (Figure 11B). Forty-eight hours after transformation, colonies were picked for inoculation of 5 mL cultures. When incubated at 30˚C with a constant agitation of 180 rpm (instead of 270 rpm), these cultures yielded the proper-sized plasmids (Figure 11C). Large-

scale cultures were grown under the same conditions. 25µg aliquots of the plasmid were stored in sterile PBS at -20˚C until electroporation.

2. The thymidine kinase protein that is encoded in the neo-tk cassette is as stable as cellular bulk protein with an estimated long half-life of around 40 hours (33), meaning that the deletion of the gene does not make the cell insensitive to ganciclovir for several days. In order to make sure that no tk is left in the cells, they were grown in selection-free medium for 5 days before ganciclovir was added. Due to this long time period, the cultures have to be split between transfection and selection. Care should therefore be taken that independent clones are generated. After the deep split, a pure ES cell clone was expanded to 25 cm^2, trypsininzed, and electroporated with 25µg FLPe plasmid. One tenth of the cells were seeded onto the wells of a 6-well plate containing wild-type mouse embryonic fibroblasts (wtMEFs). Two-three days after electroporation, the cells of each 6-well were split onto two 6-well plates at a dilution of 1:30 and 1:100. After another 2-3 days, ganciclovir selection was started.
3. Ganciclovir is a nucleotide analogue that may cause mutations. We therefore resequenced crucial sections of the recombined alleles. In the ASS allele, we did indeed find a mutation in the targeted exon that resulted in a frameshift (ATC to TTT – Ala to Val). This mutation was not present before FLPe-mediated excision and ganciclovir selection, and would have inactivated the enzymatic activity (34).
4. *Flpe*-deleter ("flipper") mice (22) are available and have already shown their usefulness in excising selection cassettes (see II.d.1, comment 3).

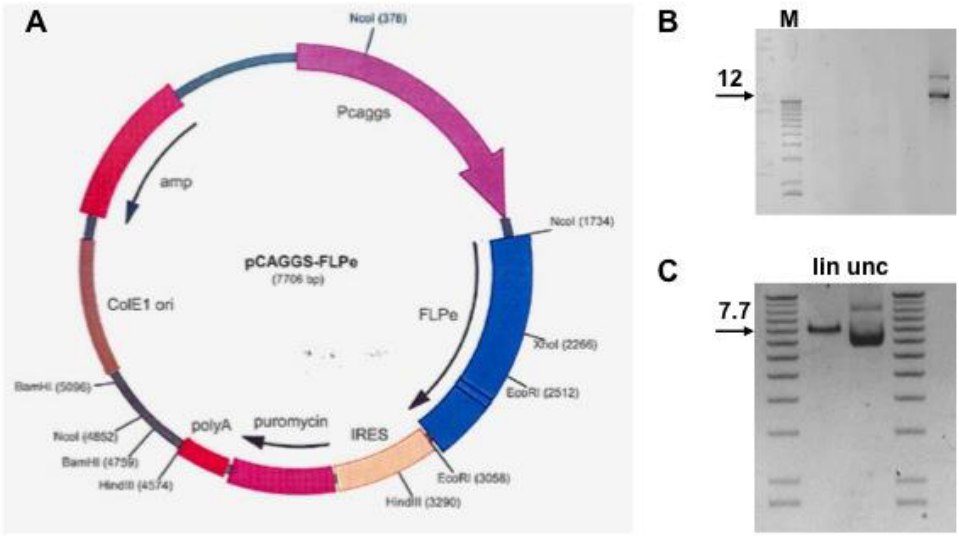

Figure 11: Flpe plasmid. Panel A: Schematic representation of the pCAGGS- expression vector. *Flpe* expression is driven by the chimeric chicken actin/CMV promoter-enhancer (18). The ampicillin (amp) and puromycin resistance genes are present for selection in bacteria and eukaryotic cells, respectively. The *flpe* sequence harbours four amino-acid changes (P2S, L33S, Y108N, S294P) that collectively improve the *in vitro* recombination activity 4-fold at 37ºC compared with wild-type *flpe* (16). Panel B: The plasmid was electroporated into XL-1 Blue bacteria, using a standard mini-preparation. The observed size of the supercoiled form of the 7.7 kb plasmid appeared to exceed 12 kb (arrow). M: Size marker. Panel C: Under optimized bacterial culture conditions (180rpm; 30˚C), the linearized (lin) plasmid was 7.7kb long (arrow) and the uncut (unc), supercoiled form ~5.5kb.

Injection of ES Cells into Blastocysts

A clone that passed all tests was karyotyped before injection into the blastocyst. Despite all quality checks, ES cells may incur damage during transfection, freezing, selection, and culture procedures, that is not detected. Therefore, the karyotype of the ES cells of each ES line was routinely tested. At least 15 metaphase spreads were counted, of which at least 80% should have the proper karyotype (40 chromosomes).

To exclude artifacts due to DNA modifications in the ES cells, germ-line chimeras of at least two independent modified clones should be generated. To eliminate DNA mutations outside the locus of interest that have accumulated during many generations of *in vitro* culture of the ES cells, newly generated genetically modified mice should be bred into wild-type mice for several generations.

Comment

Prior to blastocyst injection, the ES cells should pass the following checklist:

1. Correct DNA stoichiometry by Southern hybridization (1:1 ratio between wild-type and recombinant allele).
2. Morphology check (undifferentiated status, Figure 8B)
3. Karyotyping

Breeding Schemes

Chimeras

A rule of the thumb is that the best – in the sense of germline transmission - chimeras should be moderately chimeric. However, we do not know in advance whether the genetically modified ES cells that we produced are still sufficiently pluripotent. In the case of arginase I, the best chimeras gave 5 floxed animals out of 20-25 offspring. Since the percentage of ES cells that populates the germline is always relatively small and since an ES cell can populate the germline without carrying the modified allele, it can be very reassuring to identify a germline transmission of a modified ES cell that, as a result of meiosis, does not carry the floxed allele. This is possible based on the coat colour of the offspring.

The 129P2/HsdOla line carries the coat colour alleles A/A and c^{ch}/c^{ch}, whereas the important colour alleles of the C57Bl/6 donors of the blastocysts are a/a and C/C. A stands for agouti and is the normal brown wild-type colour of mice, whereas “a” stands for non-agouti, making the mice black. In addition, however, the c or C alleles influence the development of the colour determined by the a/A alleles. C stands for albino, meaning a c/c mouse will be white, whereas C will support whatever colour is determined by A. The allele c^{ch} stands for chinchilla and will result in

dilution of the colour determined by the A-allele, yielding beige or grey mice. An a/a, C/C mouse is a black mouse, such as the C57Bl6. An A/A, c^{ch}/c^{ch} mouse (donor of the stem cells) shows a beige coat (and red eyes). In the chimera, those cells originating from the donor blastocyst are a/a C/C, whereas those cells originating from the modified ES cells are A/A c^{ch}/c^{ch}. The fur coat therefore reveals whether offspring is chimeric.

When chimeric males are bred with C57Bl6 or 129P2/HsdOla females, the beige coat colour of the offspring will indicate that there was germline transmission of the ES cells injected into the blastocysts, independent of the demonstration of the recombinant gene. This is because the male chimera can donate either a/C (wild type) or A/c^{ch} (derived from the ES cells) sperm cells, while a C57Bl6 mother has a/C eggs. After fertilization, the offspring will either be a/a C/C (pure C57Bl6), yielding a black and therefore wild-type animal. If the offspring is A/a C/c^{ch}, resulting in a brown (agouti-coloured) animal, germ line transmission of the ES cell line has taken place.

Coat-colour can be used in a similar way to check for germline transmission in a crossing of male chimeras with 129P2/HsdOla mice. Again the male chimera provides sperm cells that are either a/C or A/c^{ch}, while the female 129P2/HsdOla has A/c^{ch} egg cells. Two types of offspring colour can be expected: a brown (agouti) animal with the genotype A/a C/c^{ch}, showing that the chimera donated a wild-type allele, or a cream-coloured, red-eyed animal (like the mother), meaning that the chimera donated the colour alleles from the injected ES-cells. Therefore, cream-coloured animals are evidence for germline transmission.

Animals with a proper coat colour still need to be genotyped for genetic modification, because crossing over may segregate the floxed allele from those conferring coat colours. Nevertheless, an animal with a proper coat colour shows that the chimeric male that sired, is a promising candidate for offspring carrying the floxed allele. A chimeric mouse that only yields black offspring (>20 pubs) should not be used for further breedings. Furthermore, even when brown or light-brown offspring are obtained, one might have to analyze several nests before ever getting a positive offspring showing germline transmission. For the ASS project, a chimeric male breeding with Ola females gave 10 nests with a total of 61 offspring. Although the offspring were predominantly brown, the first 52 offspring (first 9 nests) gave no positive animals for the ASS construct. We had to wait for the 10^{th} nest to get one mouse positive for the ASS construct.

There are actually more alleles determining coat colour, that these alleles do not play a role here. Depending on the ES cell line used, it may be worthwhile to check the genetics beforehand and use this information to decide on crossings that yield a 2-fold higher rate of ES-cell derived offspring. A comprehensive review of coat colour alleles and their interaction can be found on www.jax.org by Willys K. Silvers (1979), The coat Colors of Mice; a model for mammalian gene action and interaction**.**

Breeding tissue-specific knockout mice

The ES cells injected into the blastocysts have one floxed allele and one wild-type allele. The chimeras obtained are bred to find offspring that carry the floxed genotype in their germline. This mouse is then bred to increase the number of floxed offspring. It is also crossed with a Cre mouse, in which *Cre* is expressed under control of the EIIa promoter. This promoter is expressed at the zygotic stage, i.e. before the germline is set aside, so that we obtain a hemizygous knockout mouse (35). This hemizygous knockout will be crossed with hemizygous floxed mice.

It takes at least three generations of mice from the chimera to the genetically modified animal (Figure 12). If we first work with a floxed allele and a tissue-specific *cre*, we will go for a safe breeding protocol, meaning that $X^{flox/flox}$ mice are crossed with $X^{+/-}/Cre^{+/-}$ mice. The advantage of this approach is that *Cre* has to inactivate only one allele in the tissue of interest, doubling the chance that the cells in the tissue of interest do not express the gene X. The disadvantage of this approach is that all cells in the body only contain 1 functional allele of gene X and, hence, usually only 50% of the normal protein content. If excision proves efficient, we prefer matings between $X^{flox/flox}$ and $X^{flox/flox}/Cre^{+/-}$ mice, because all cells in these mice, where Cre is not active, contain 2 functional copies of gene X.

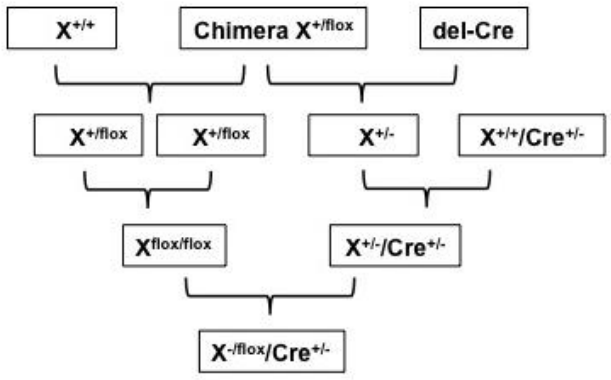

Figure 12: Breeding scheme to generate tissue-specific knockout mice. Chimeras are bred with wild-type mice to obtain mice with 100% germline transmission and with "del-*Cre*" mice to obtain mice, which are heterozygous for the target gene. The resulting $X^{+/flox}$ mice (left arm of scheme) are interbred to obtain $X^{flox/flox}$ mice, which can be produced with a high efficiency. The heterozygous $X^{+/-}$ mice (right arm of scheme) are bred with the desired tissue-specific *Cre* mice to generate $X^{+/-}/Cre^{+/-}$ mice. These mice are then crossed with the $X^{flox/flox}$ mice to obtain $X^{-/flox}/Cre^{+/-}$ mice, which have gene X deleted in a tissue-specific manner. This scheme is advisable if *Cre* is suspected to remove the floxed fragment inefficiently. However, all cells in the organism have the $X^{+/-}$ genotype. If *Cre* efficiently removes floxed fragments, one can better use mice with a $X^{flox/flox}/Cre^{+/-}$ genotype, because then the targeted sequence is deleted in the tissue of interest only.

Conclusions

The following are the important factors to be considered from our experience.

1. **Constructing the targeting vector:** If using the PCR-based approach to amplify the homologous arms from genomic DNA, BAC clones should be used to decrease the number of amplications. Furthermore, repeats in the regions to be amplified should be avoided as they are very difficult to amplify by PCR. Finally, sequence differences between strains should be taken into account when selecting the homologous sequences:

they may interfere with the efficiency of the homologous recombination and should not be interpreted as mutations.

2. **Selection of the recombinant ES clone**: The clone to be injected into blastocysts should contain only homologously recombined cells. To achieve this, selected colonies should be continuously cultured in presence of the appropriate selection drug to avoid the growth of wild-type cells.
3. **Screening against random integration**: The "short" PCR method to eliminate the randomly integrated clones described in III.a. is an easy, fast and reliable method to eliminate randomly integrated clones, if the target DNA is not be located totally at the end of the constructs.
4. **Screening for homologous recombination**: The "long" PCR procedure to identify and characterize recombination events is faster and more informative than Southern blots. A draw back is that the primer pair cannot be tested and optimized before the recombinant clone is available.
5. **Selection of probes for Southern blotting**: Intronic probes have to be checked for the presence of repeats. A 300bp probe can suffice for sensitive and specific detection.
6. **Selection with ganciclovir**: Ganciclovir causes mutations. It is therefore necessary to resequence the clone after the excision of the selection cassette.
7. **Coat colour as a marker for germline transmission:** Coat colour as a marker doubles the chance to identify a male with proper germline transmission of the floxed allele.
8. **Breeding set-up:** The best genotypes to study physiological effects of gene knock out are flox/flox and flox/flox-Cre. In all our modified mice (ASS, Arg-1 and CAT-1), we obtained very good excision efficiency *in vivo* of both alleles with *cre*. It is therefore recommended to set up breeding schemes that yield these genotypes.

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CHAPTER III

ABLATION OF *Arg1* IN HEMATOPOEITIC CELLS IMPROVES RESPIRATORY FUNCTION OF LUNG PARENCHYMA, BUT NOT THAT OF LARGER AIRWAYS OR INFLAMMATION IN ASTHMATIC MICE

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Ablation of *Arg1* in hematopoietic cells improves respiratory function of lung parenchyma, but not that of larger airways or inflammation in asthmatic mice

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Cloots RH, Sankaranarayanan S, de Theije CC, Poynter ME, Terwindt E, van Dijk P, Hakvoort TB, Lamers WH, Köhler SE. Ablation of *Arg1* in hematopoietic cells improves respiratory function of lung parenchyma, but not that of larger airways or inflammation in asthmatic mice. *Am J Physiol Lung Cell Mol Physiol* 305: L364–L376, 2013. First published July 5, 2013; doi:10.1152/ajplung.00341.2012.—Asthma is a chronic inflammatory disease of the small airways, with airway hyperresponsiveness (AHR) and inflammation as hallmarks. Recent studies suggest a role for arginase in asthma pathogenesis, possibly because arginine is the substrate for both arginase and NO synthase and because NO modulates bronchial tone and inflammation. Our objective was to investigate the importance of increased pulmonary arginase 1 expression on methacholine-induced AHR and lung inflammation in a mouse model of allergic asthma. Arginase 1 expression in the lung was ablated by crossing $Arg1^{fl/fl}$ with $Tie2Cre^{tg/-}$ mice. Mice were sensitized and then challenged with ovalbumin. Lung function was measured with the Flexivent. Adaptive changes in gene expression, chemokine and cytokine secretion, and lung histology were quantified with quantitative PCR, ELISA, and immunohistochemistry. *Arg1* deficiency did not affect the allergic response in lungs and large-airway resistance, but it improved peripheral lung function (tissue elastance and resistance) and attenuated adaptive increases in mRNA expression of arginine-catabolizing enzymes *Arg2* and *Nos2*, arginine transporters *Slc7a1* and *Slc7a7*, chemokines *Ccl2* and *Ccl11*, cytokines *Tnfa* and *Ifng*, mucus-associated epithelial markers *Clca3* and *Muc5ac*, and lung content of IL-13 and CCL11. However, expression of *Il4*, *Il5*, *Il10*, and *Il13* mRNA; lung content of IL-4, IL-5, IL-10, TNF-α, and IFN-γ protein; and lung pathology were not affected. Correlation analysis showed that *Arg1* ablation disturbed the coordinated pulmonary response to ovalbumin challenges, suggesting arginine (metabolite) dependence of this response. *Arg1* ablation in the lung improved peripheral lung function and affected arginine metabolism but had little effect on airway inflammation.

airway hyperresponsiveness; arginine; inflammation

ALLERGIC ASTHMA IS A CHRONIC inflammatory disorder of the lung that is characterized by a reversible limitation of the airflow due to an allergic reaction in the airways with the characteristics of a T_H2-predominant airway inflammation. The airway inflammation is initiated by the binding of inhaled allergens to complementary IgEs on IgE receptor-bearing cells. Upon activation, these cells release histamine, cytokines, and proteases, resulting in the activation of the inflammatory cascade with lung infiltration of eosinophils and mononuclear cells. Increased mucus production, mucosal edema, and a disproportionate smooth-muscle contraction lead to airway hyperresponsiveness (AHR). As a result of the recurring inflammation, airway remodeling develops as a consequence of fibrosis and airway wall thickening (2, 3, 6, 36).

Recent studies in animal models and patients revealed a potential role for the arginine-catabolizing enzyme arginase in the pathogenesis of asthma (28, 53). Arginase exists as two isoforms, arginase 1 and arginase 2. The expression of cytoplasmic arginase 1 and mitochondrial arginase 2 is undetectable in healthy mouse lungs but is strongly upregulated in mouse models of allergic asthma (50, 59). Arginase 1 is only found in cells with the morphological characteristics of macrophages that are present in the inflammatory infiltrates of asthmatic lungs (50, 59). Arginases catabolize L-arginine to urea and ornithine, but arginine is also the substrate for nitric oxide (NO) synthesis by NO synthase (NOS). A deficient NO production represents a key feature of AHR (36), since NO modulates bronchial and vascular tone by inducing relaxation of smooth muscle cells. NOS enzymes have a higher affinity for arginine (K_m: 2–20 μM) than arginases (K_m: 1–5 mM), but the V_{max} of arginase is ~1,000-fold higher than that of NOS, which confers the capability for regional substrate depletion of arginine (56).

The role of arginases in the development and symptoms of allergic asthma is still incompletely understood. We, therefore, examined the effects of *Arg1* deletion in the lung on methacholine-induced AHR and lung inflammation in a mouse model of asthma. In rodents, extensive arginase 1 expression in the myeloid lineage of blood cells is confined to macrophages and dendritic cells (37). For that reason, we generated cell-specific arginase 1 knockouts by flanking exon 4 of *Arg1* with loxP sites and crossing $Arg1^{fl/fl}$ mice with either $LysMCre^{tg/-}$ (9) or $Tie2Cre^{tg/-}$ mice (23) to delete *Arg1* in myeloid cells (9), or in all hematopoietic and endothelial cells (47), respectively. We sensitized and then exposed these mice to ovalbumin to induce allergic asthma (26) and measured their responsiveness to increasing doses of methacholine. The adaptive changes in gene expression, chemokine and cytokine secretion, and lung histology were quantified. We report that selective *Arg1* ablation improves peripheral lung function, without affecting lung inflammation.

MATERIALS AND METHODS

Plasmid construction and recombinant ES cell selection. The mouse *Arg1* gene is located on chromosome 10. Since deletion of exon 4 causes a frame shift, we targeted this exon. The targeting

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vector (17.515 kb; Fig. 1*A*) consisted of *Arg1* exons and introns 2 and 3 (4.6 kb) at the 5′ end, a Neo-TK selection cassette flanked by *frt* sites (10), exon 4 (160 bp) flanked by *loxP* sites (46), introns 4–7, exons 5–8, and a small fragment downstream of the gene (in total 4.3 kb) at the 3′ end. The targeting construct was sequence verified with respect to exons, splice junctions, and recombinase-recognition sites; digested with *Asc*I (introduced by PCR for cloning purposes); and purified by electrophoresis and electroelution. Twenty-five micrograms of the targeting fragment was electroporated into the mouse ES cell line E14IB10 (129/Ola). Selection with G418 (200 μg/ml) was started 24 h after electroporation. Vector sequences that were left on either end of the targeting construct allowed a PCR-based negative screen against random integrations. Proper recombination of the 3′ end was demonstrated with Southern blotting after Blp1 digestion (Fig. 1*A*) and probing with a 300-bp external genomic fragment, and by long-distance PCR, using AccuTaq (Sigma), an *Arg1*-specific primer 3′ outside the targeting construct and a *loxP*-specific primer (Fig. 1*A*). Proper recombination of the 5′ end was demonstrated by long-distance PCR, using AccuTaq, a 5′ *frt*-specific primer, and an *Arg1*-specific primer 5′ outside the targeting construct (Fig. 1*A*). Exons and junctions of candidate ES clones were sequence verified by using those long-PCR products. After a "deep split" to ensure the presence of recombined ES clones only (the recombined 8.5-kb band in the initial isolate was much less intense than the endogenous 6.1 kb band), the Neo-TK cassette was removed by transient transfection with an FLPe recombinase expression vector (kindly provided by Dr.

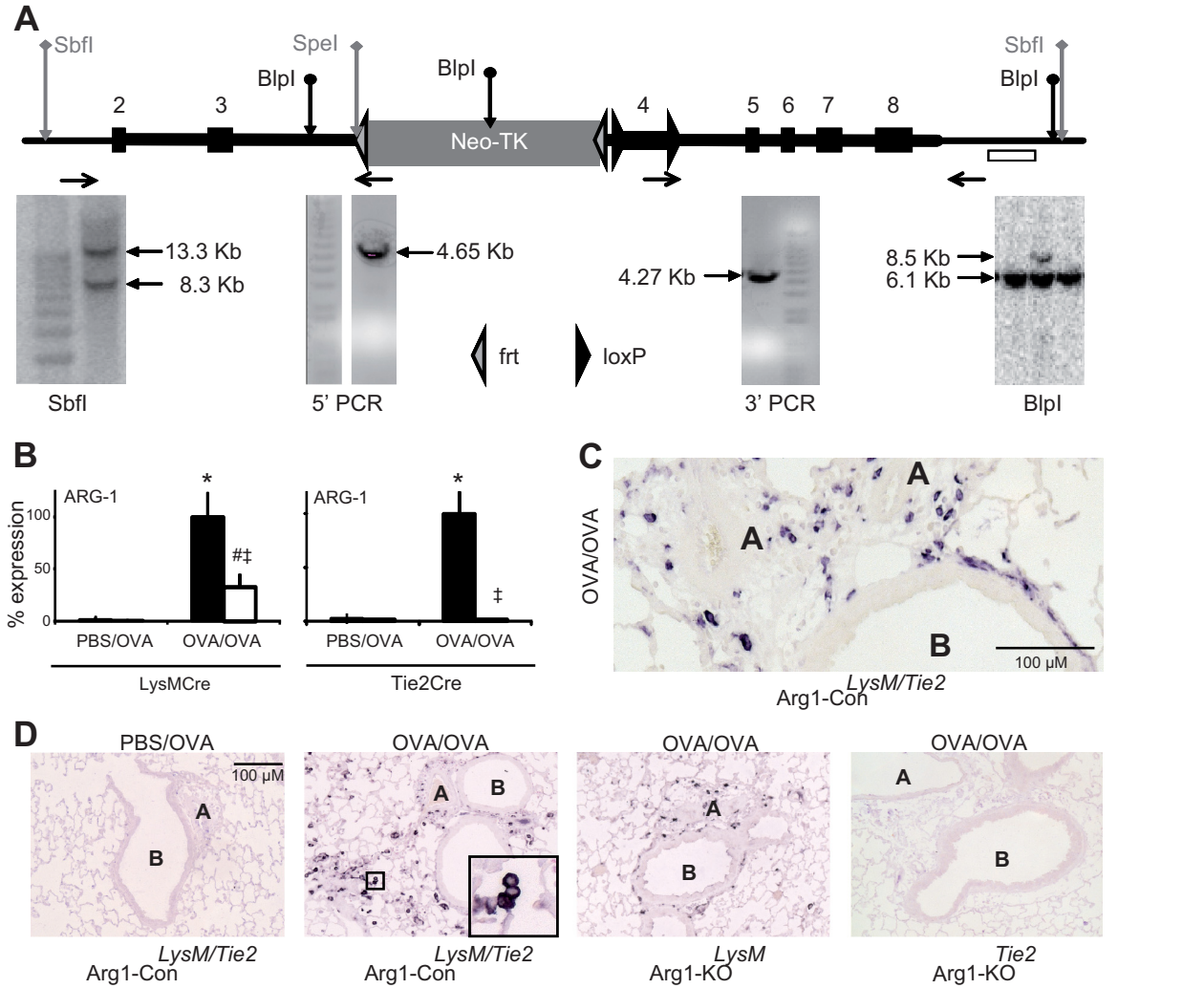

Fig. 1. Construction of $Arg1^{fl/fl}$ mice and validation of *Arg1* elimination in lung. *A*: targeting construct of murine *Arg1* gene. The *Frt*-flanked Neo-TK selection cassette in intron 3, floxed exon 4, and the positions of relevant restriction enzymes are indicated. Blp1 was used to differentiate the recombined allele (8.5 kb) from the wild-type allele (6.1 kb; right Southern blot); Sbf1 and *Spe*I, both introduced during cloning, were used to demonstrate a 1:1 ratio between the recombined (8.3 kb) and wild-type (13.3 kb) *Arg1* alleles in the blastocyst-injected ES cells (*left* Southern blot). The 3′ 300-bp DNA fragment used as Southern probe is shown as a white box. The products of the 5′ and 3′ long-distance PCRs (4.65 and 4.27 kb, respectively), used to validate the sequence of the recombined allele, are shown in between the Southern blots. Note that in the picture of the 5′ PCR 3 empty lanes were removed between the marker lane and the lane with the 5′ PCR product. *B*: reduced arginase 1 protein levels in the lungs of ovalbumin (OVA)/OVA-treated Arg1-KO^{LysM} ($n = 9$) or Arg1-KO^{Tie2} mice ($n = 9$), open bars, compared with the corresponding Arg1-Con mice (solid bars). *C*: detail of arginase 1 expression in OVA/OVA-treated Arg1-Con lung. Note absence of expression in arterial endothelium. A, artery; B, bronchus. Bar: 100 μm. *D*: sections of PBS/OVA- and OVA/OVA-treated lungs of Arg1-Con mice (*left* 2 panels) and of OVA/OVA-treated $Arg1^{fl/fl}/LysMCre^{tg/-}$ (Arg1-KO^{LysM}) and $Arg1^{fl/fl}/Tie2Cre^{tg/-}$ (Arg1-KO^{Tie2}) mice (*right* 2 panels) stained immunohistochemically for the presence of arginase 1-positive cells. Note reduction of arginase 1-positive cells in Arg1-KO^{LysM} mice and absence in Arg1-KO^{Tie2} mice. $^*P < 0.05$ OVA/OVA vs. PBS/OVA Arg1-$Con^{LysM/Tie2}$; #$P < 0.05$ OVA/OVA vs. PBS/OVA Arg1-$KO^{LysM/Tie2}$; ‡$P < 0.05$ OVA/OVA Arg1-$Con^{LysM/Tie2}$ vs. Arg1-$KO^{LysM/Tie2}$.

Francis Stewart, Biotechnology Center, TU Dresden, Germany). Selection with ganciclovir (5 μM) was started 5 days after electroporation. The recombined allele was again sequence verified [ganciclovir introduces mutations in ~25% of clones (our unpublished observations)]. Two clones with 40 chromosomes and a 1:1 ratio of wild-type and recombined *Arg1* alleles [tested after digestion with Sbf1 (restriction sites outside targeting construct) and Spe1 (sites present in targeting construct only) and hybridization with the 300-bp external genomic fragment] (Fig. 1*A*) were selected for blastocyst injection.

Generation of transgenic mice and husbandry. All animal experiments were approved by the Committee for Animal Care and Use of Maastricht University. *Arg1*-recombinant ES cells were injected into C57BL/6J blastocysts. Chimeric male mice were bred with female 129P2/OlaHsd mice (Harlan, The Netherlands) and with deleter-Cre females on a C57BL/6J background (48; kindly provided by Dr. Ursula Lichtenberg, University of Cologne, Cologne, Germany) to delete the *Arg1* exon 4 in the germ line. Mice were genotyped with primers Arg1-F1 and Arg1-R1 (Table 1), yielding a 351-bp wild-type allele and a 384-bp floxed allele. The Cre-excised allele (298 bp) was detected by PCR with the primers Arg1-F2 (Table 1) and Arg1-R1. To specifically delete the floxed *Arg1* allele ($Arg1^{fl}$) in macrophages, mice were crossed with $LysMCre^{tg/-}$(9) or $Tie2Cre^{tg/-}$ mice (23). The $Tie2Cre^{tg/-}$ and $LysMCre^{tg/-}$ mice are available from Jackson Laboratories as B6.Cg-Tg$^{(TekCre)1Ywa}$/J and B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J mice, respectively. The resulting $Arg1^{fl/fl}/LysMCre^{tg/-}$ (Arg1-KO^{LysM}) mice and their $Arg1^{fl/fl}/LysMCre^{-/-}$ littermates (Arg1-Con) or $Arg1^{fl/fl}/Tie2Cre^{tg/-}$ (Arg1-KO^{Tie2}) mice and $Arg1^{fl/fl}/Tie2Cre^{-/-}$ littermates [Arg1-Con were genotyped for the presence of LysMCre or Tie2Cre by PCR with primers LysM-F and LysM-R or Tie2-F and Tie2-R (Table 1)], yielding 300-bp- or 510-bp-positive bands, respectively, for Cre-positive animals. Arg1-Con and Arg1-KO^{LysM} were 75% C57BL/6J and 25% 129P2OlaHsd, whereas Arg1-Con and Arg1-KO^{Tie2} were 50% C57BL/6J, 25% 129P2OlaHsd, and 25% FVB.

Table 1. *Primers pairs used for genotyping and quantitative PCR*

Gene	Primer Name	Primer Sequence (5′-3′)
Excised *arginase-1*	Arg1-F2	TCTAGAACTAGTGGATCACCTCAG
	Arg1-R1	GTGCCTTGGTCTACATTGAACATAC
Cre	CRE-F	GGTTCGCAAGAACCTGATGGACAT
	CRE-R	GCTAGAGCCTGTTTTGCACGTTCA
Tie-2-Cre	Tie2-F	CGCATAACCAGTGAAACAGCATTGC
	Tie2-R	CCCTGTGCTCAGACAGAAATGAGA
LysMCre	LysM-F	GGTTCGCAAGAACCTGATGGACAT
	LysM-R	GCTAGAGCCTGTTTTGCACGTTCA
Arginase-1	Arg1-F1	GGAGAGCCTTCCTGCACTTT
	Arg1-R1	GTGCCTTGGTCTACATTGAACATAC
Arginase-2	Arg2-F	CCAGCTGCCATTCGAGAAG
	Arg2-R	ATCATCTTGTGGGACATTAGTAAACTC
Nos-2	Nos2-F	GCCACCAACAATGGCAACA
	Nos2-R	CGTACCGGATGAGCTGTGAATT
Scl7a1	Cat1-F	CTGGTGGACCTCATGTCCATT
	Cat1-R	GCTCATTCTGATCTACTCGATCTAGCT
Slc7a2b	Cat2b-F	GATCCATTTTCCCAATGCCTC
	Cat2b-R	TGGAATTGATTTGAGCTAGACATTTG
Slc7a7	Lat1-F	GAAGGACCCCGACCGG
	Lat1-R	AACAGCCACCAGGAAGATGG
Il4	IL4-F	TGGAATGTACCAGGAGCCATATCC
	IL4-R	CTCTGTGGTGTTCTTCGTTGCTGT
Il13	IL13-F	CACACAAGACCAGACTCCCCTG
	IL13-R	GGTTACAGAGGCCATGCAATATCC
Il5	IL5-F	ATCAAACTGTCCGTGGGGGTACT
	IL5-R	TCTCTCCTCGGCCACACTTCTCTTTT
Il10	IL10-F	GGACAACATACTGCTAACCGACTCCT
	IL10-R	CTGCTCCACTGCCTTGCTCTTATT
Ccl2	MCP1-F	GCTGGAGAGCTACAAGAGGAT
	MCP1-R	ACAGACCTCTCTCTTGAGCTTGGT
Ccl11	Eotaxin1-F	CTGCTGCTCACGGTCACTTCCT
	Eotaxin1-R	CAGGGTGCATCTGTTGTTGGTG
Ifng	IFNG-F	GGTTGCTCCTCTTACCGTCTTT
	IFNG-R	CGTGGCACTTTTTACCACAGA
Tnfa	TNFA-F	TCAATCGGCCCGACTATCTC
	TNFA-R	CAGGGCAATTGATCCCAAAGT
Muc5ac	MUC5AC-F	GATGACTTCCAGACTATCAGTG
	MUC5AC-R	TGGCGTTAGTCAGCAGA
Clca3	CLCA3-F	CCGGCTGCCGCTAAAGAG
	CLCA3-R	CAGAAGCATCAACAAGACCATTG
18S	18S-F	AGTTAGCATGCCAGAGTCTCG
	18S-R	TGCATGGCCGTTCTTAGTTG

Antigen sensitization and challenge. Ten-week-old male Arg1-KO^{LysM} mice or Arg1-KO^{Tie2} mice, and their Arg1-Con littermates were injected intraperitoneally on days 0 and 14 with 10 μg of ovalbumin (OVA), grade V (Sigma-Aldrich, Zwijndrecht, The Netherlands) in a total volume of 100 μl PBS, containing 1 mg/ml of $AlOH_3$ (alum) as adjuvant (Imject Alum, Thermo Scientific, Rockford, IL). On *days 21–27*, mice were exposed daily for 30 min to 1% (wt/vol) aerosolized OVA in PBS in custom-made inhalation chambers. The experimental groups consisted of two to three mice per genotype and treatment and the experiments were repeated on at least three different time points. AHR was assessed 12 h after the last challenge (41). Lung airway resistance R_N, tissue elastance H, and tissue resistance G in response to inhaled aerosolized methacholine were measured by use of a Flexivent apparatus (Scireq, Montreal, Canada).

Airway hyperresponsiveness. Mice were anesthetized with pentobarbital sodium. A total dose of 120 mg/kg was administered per mouse. First 80 mg/kg was administered to induce anesthesia and 40 mg/kg ~30 min later to maintain anesthesia. An 18-gauge blunt needle was inserted into the trachea and connected to a mechanical small animal ventilator (FlexiVent, Scireq, Montreal, Canada). Mice were ventilated at 200 breaths/min with a delivered tidal volume of 0.25 ml against a positive end-expiratory pressure of 3 cmH_2O applied by a water trap for 10 min before the experiment was started. Aerosolized methacholine (Sigma, Steinheim, Germany) challenges were performed by delivering successively 0, 3.1, 12.5, and 50 mg/ml methacholine in PBS. Following each methacholine challenge, ventilation was interrupted every 10 s to allow for 1-s passive expiration followed by 2-s broad-band (1–19.6 Hz) volume perturbation. The peak-to-peak excursion of the ventilator piston during delivery of these perturbations was 0.17 ml, resulting in a delivery of ~0.14 ml after correcting for gas compression in the ventilator cylinder and connecting tubing. Pressure and flow were recorded during application of the perturbations and used to calculate the input impedance (Zrs) of the respiratory system. Zrs was then fitted to the uniformly ventilated model of the lung with constant-phase tissue impedance (12, 16).

$$Z_{rs}(f) = R_N + i2\pi f I_{aw} + \frac{G - iH}{(2\pi f)^a}$$

R_N is the Newtonian resistance composed mostly of the flow resistance of the conducting pulmonary airways; I_{aw} is the inertance of the gas in the central airways; H, also known as elastance, reflects the elastic energy stored in the tissues; G, also known as tissue resistance, reflects viscous dissipation of energy in the respiratory tissues as well as airflow heterogeneity; f is frequency; $i = \sqrt{-1}$; and α couples G and H. R_N, G, and H all have units of $cmH_2O{\cdot}s{\cdot}ml^{-1}$. R_N and G are shown as maximal resistance and tissue damping after application of the methacholine challenge, whereas H is shown as the plateau value of the elastance following the initial peak response after the methacholine challenge.

Plasma collection and analysis. At the end of the experiment, blood was collected from the inferior caval vein in heparin-coated tubes, centrifuged for 3 min at 5,000 *g*, snap frozen in liquid nitrogen, and stored at −80°C. Plasma OVA-specific IgE levels were deter-

Table 2. *Amino acid concentrations in venous plasma of 8-day-old Arg1- KO^{delCre}*

Amino Acid	Arg1-Con	Arg1-KO^{delCre}
Glu	115 ± 8	210 ± 49*
Asn	97 ± 3	138 ± 33
Ser	361 ± 19	327 ± 85
Gln	611 ± 26	1344 ± 279*
His	143 ± 7	200 ± 24
Gly	417 ± 23	326 ± 61
Thr	293 ± 9	276 ± 57
Cit	94 ± 3	176 ± 12*
Arg	251 ± 13	2185 ± 295*
Ala	452 ± 31	303 ± 120
Tyr	190 ± 8	211 ± 68
Tau	511 ± 55	743 ± 96
Val	322 ± 24	300 ± 69
Met	126 ± 3	234 ± 52
Ile	125 ± 11	142 ± 26
Phe	106 ± 5	82 ± 9*
Orn	175 ± 16	112 ± 30
Leu	191 ± 13	206 ± 46
Trp	92 ± 2	68 ± 10*
Lys	833 ± 38	404 ± 31*
Total amino acids	5,506 ± 208	7,987 ± 1259

Values are μM ± SE. *$P < 0.05$ Arg1-Con^{delCre} (*Arg1$^{+/fl}$/delCre$^{-/-}$*) vs. Arg1-KO^{delCre} (*Arg1$^{-/fl}$/delCre$^{tg/-}$*).

mined by ELISA (MD Biosciences, M036005, Zürich, Switzerland). For the determination of plasma amino acids, 50 μl of plasma was added to 4 mg sulfosalicylic acid, vortexed, snap frozen in liquid nitrogen, and stored at −80°C until use. Plasma amino acid concentrations were measured with a fully automated HPLC system (54).

Tissue isolation. Immediately following euthanasia, lungs were isolated. The left lung was filled with 4% formaldehyde (Klinipath, Deventer, The Netherlands) for 10 min at a pressure of 20 cmH_2O and submersed overnight in 4% formaldehyde at room temperature (RT) prior to paraffin embedding. The right lung was snap frozen in liquid nitrogen and pulverized in a liquid-nitrogen-chilled mortar and pestle. Tissue powder was stored at −80°C until further use.

Histology and immunohistochemistry. Paraffin-embedded tissue was cut into 4 μm thick sections and stained with hematoxylin & eosin (H&E), Sirius red (collagen fibers), or ammoniacal silver nitrate (reticular fibers). For immunostaining, epitopes were retrieved by heating the slides for 5 min in 10 mM sodium citrate (pH 6) at 95°C and cooling to RT for 30 min before blocking endogenous peroxidases with peroxidase block (DAKO, S2001, Enschede, the Netherlands) for 10 min at RT. This step was omitted if antibody binding was visualized with the alkaline phosphatase (AP) system. Sections were blocked with 10% normal goat serum for 30 min. After washing in PBS, slides were incubated with anti-arginase 1 (Amsterdam Liver Center, AMS40.11.13), anti-arginase 2 (sc-20151Santa Cruz Biotechnology, California), anti-myeloperoxidase (MPO; DAKO), or anti-major basic protein (MBP; kindly provided by Dr. James Lee, MT14.3.7, Mayo Clinic Scottsdale, AZ) (27). After washing, sections were incubated with a 1:200 diluted biotinylated rabbit anti-rat secondary antibody (DAKO) for 45 min at RT. Sections were washed, incubated with streptavidin/horseradish peroxidase (HRP; Vector) for 30 min at RT and developed with 3,3′-diaminobenzidine (Sigma, Steinheim, Germany) for 10 min. Sections stained for arginase 1 and 2 were incubated with an AP-labeled secondary antibody (1:200, Sigma A-3687) for 45 min, developed for 30 min with nitroblue-tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche, Almere, The Netherlands), dissolved in 50 mM $MgSO_4$, 100 mM Tris·HCl (pH 9.5), and coverslipped with an aqueous mounting medium (DAKO).

Histological assessment. The H&E-stained slides were used to identify inflammation in general. Sirius red- and silver nitrate-stained slides, which visualize collagen and reticular fibers, respectively, were used to assess tissue remodeling in the lungs of OVA/OVA-treated mice. MBP-, MPO-, and ARG2-positive cells in full-face sections of the lung were imaged with a panoramic 3DHISTECH slide scanner and counted with 3DHISTECH software (3DHISTECH, Budapest,

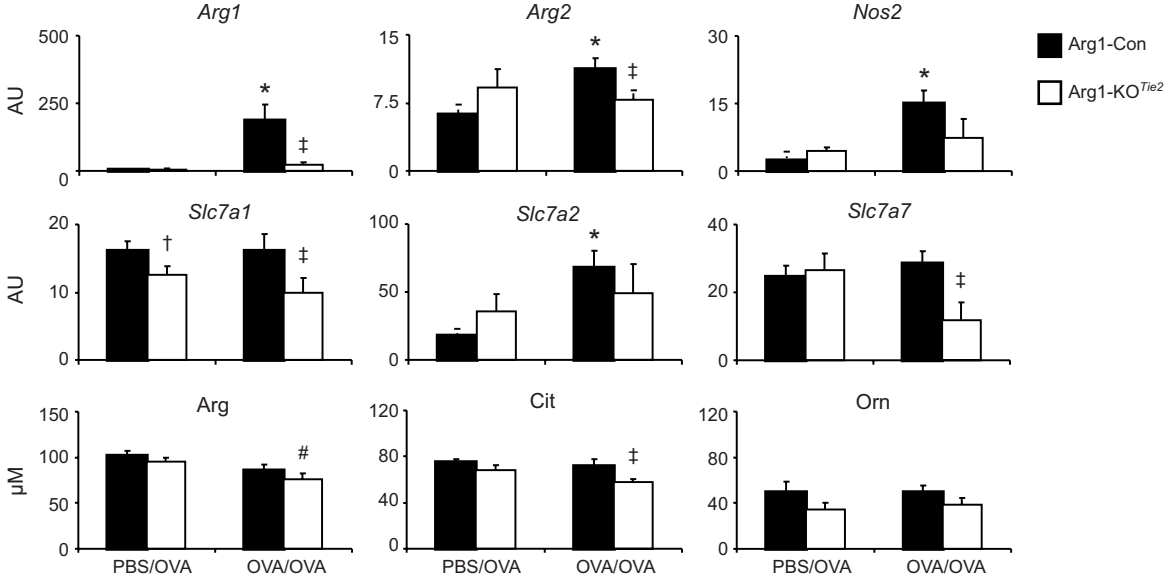

Fig. 2. Expression of arginine-metabolizing enzymes and transporters in mouse lungs that are or are not *Arg1* deficient. Arg1-Con and Arg1-KO^{Tie2} are represented by solid and open bars, respectively. Treatment (PBS/OVA or OVA/OVA) is indicated below the bars. The mRNA abundance (AU; number of mRNA copies normalized for 18S rRNA expression and multiplied by 10,000) of the arginine-metabolizing enzymes *Arg1*, *Arg2*, and *Nos2* and the arginine transporters *Slc7a1*, *Slc7a2*, and *Slc7a7* is shown as means ± SE of 7–8 mice per group. Amino acid concentrations (μM) of arginine, citrulline, and ornithine are shown as means ± SE (PBS/OVA-treated Arg1-Con: $n = 7$; Arg1-KO^{Tie2}; $n = 8$; OVA/OVA-treated Arg1-Con: $n = 10$; Arg1-KO^{Tie2}; $n = 9$). *$P < 0.05$ PBS/OVA vs. OVA/OVA Arg1-Con; †$P < 0.05$ PBS/OVA Arg1-KO^{Tie2} vs. Arg1-Con, #$P < 0.05$ OVA/OVA vs. PBS/OVA Arg1-KO^{Tie2}; ‡$P < 0.05$ OVA/OVA Arg1-Con vs. Arg1-KO^{Tie2}.

Hungary). Detection limits were set to count cells positive for diaminobenzidine with a diameter between 10 and 15 μm. The density of cells is expressed per mm^2.

Milliplex assay. Tissue powder was homogenized in PBS, pH 7.6, in the presence of a proteinase inhibitor cocktail (Complete, Roche). Cytokines [CCL11 (a.k.a. eotaxin-1), IL-4, IL-5, IL-10, IL-13, TNF-α, and IFN-γ] were quantified by using a Luminex xMAP multiplex platform, combined with a customized Milliplex mouse chemokine/cytokine panel from Millipore (Billerica, MA).

RNA isolation and quantification. Tissue powder was homogenized in Tri reagent (Sigma) with the Mini Bead-Beater (Biospec Products, Bartlesville, OK). To remove genomic DNA, RNA was precipitated with 2 M LiCl for at least 30 min at −20°C. RNA integrity was checked by denaturing gel electrophoresis. RNA concentration was determined with a NanoDrop-ND-1000 spectrophotometer at 260 nm (Isogen Life Sciences, Wilmington, DE), and 400 ng of total RNA was transcribed using a first-strand synthesis kit (Roche). Quantitative PCR was performed in a Lightcycler 480 (Roche), using Lightcycler 480 SYBRgreen Master Mix (Roche) and the following settings: denaturation 30 s at 95°C, annealing 30 s at 60°C, elongation 30 s at 72°C, 45 cycles, and a final elongation step for 5 min at 72°C. If reverse transcriptase was omitted, no product was formed. Primary fluorescent data were exported and analyzed with the Lin-Reg Analysis program (44). mRNA abundance was expressed relative to *18S* rRNA abundance. Primer sequences are given in Table 1.

Western blot. Tissue powder was homogenized in RIPA buffer [25 mM Tris·HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% Na-deoxycholate, 0.1% SDS, containing Complete cocktail (Roche)]. Protein concentration was measured with the bicinchoninic-acid assay (Pierce, Rockford, IL). Twenty-five micrograms protein was separated on an SDS-polyacrylamide gel, transferred onto 0.45 μm nitrocellulose membranes by use of a wet transfer system (Bio-Rad, Hercules, CA), stained with Ponceau S to confirm equal loading of lanes, washed with TBS (50 mM Tris, 150 mM NaCl, pH 7.6), and blocked with 5% skimmed milk in TBS/0.5% Tween-2. Arginase 1 was visualized with rabbit anti-arginase 1 antibody (1:200), followed by an HRP-conjugated swine anti-rabbit secondary antibody (DAKO). The signal was developed using the Super Signal West Pico Substrate (Pierce) and quantified with the Fuji systems darkbox (Fuji Film Life Sciences, Tokyo, Japan).

Statistical analyses. Statistical analysis of the groups was performed using the Kruskal-Wallis test for PBS/OVA- vs. OVA/OVA-

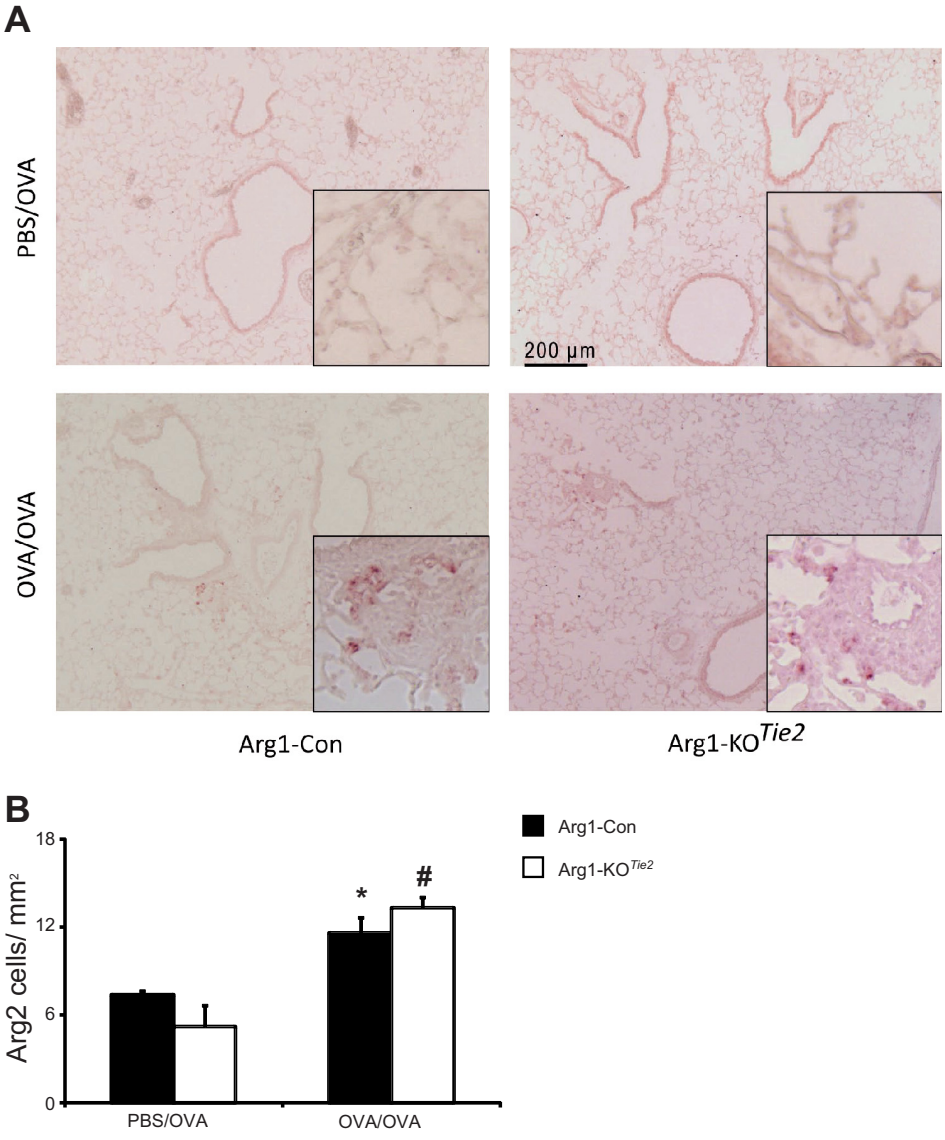

Fig. 3. Arginase 2 expression in mouse lungs that are or are not *Arg1* deficient. *A*: sections of PBS/OVA- (*top row*) and OVA/OVA-treated (*bottom row*) lungs of Arg1-Con mice (*left* 2 panels) and of Arg1-KO^{Tie2} mice (*right* 2 panels) stained immunohistochemically for the presence of arginase 2-positive cells. Main panels, ×10 objective; insets, ×40 objective. Note the increase of arginase 2-positive cells in OVA/OVA-treated mice without difference between Arg1-Con and Arg1-KO^{Tie2} mice. Bar: 200 μm. *B*: quantification of arginase 2-positive cells per mm^2 lung tissue in PBS/OVA- and OVA/OVA-treated Arg1-Con (solid bars) and Arg1-KO^{Tie2} mice (open bars). Sections were stained simultaneously. Values are shown as means ± SE (PBS/OVA-treated Arg1-Con: $n = 7$; Arg1-KO^{Tie2}; $n = 8$; OVA/OVA-treated Arg1-Con: $n = 10$; Arg1-KO^{Tie2}; $n = 9$). *$P < 0.05$ PBS/OVA vs. OVA/OVA Arg1-Con; #$P < 0.05$ OVA/OVA vs. PBS/OVA Arg1-KO^{Tie2}.

treated, and Arg1-Con vs. Arg1-KO mice. Only if this nonparametric test indicated the presence of a difference among experimental groups, a multiple comparison of the groups was carried out. Values were considered statistical significant if $P < 0.05$, and as indicating a trend if $P < 0.1$.

Pearson's bivariate two-tailed correlation coefficients between each of the lung function parameters, mRNA and protein concentrations, histology scores, and plasma amino-acid concentrations were determined after combining the data from the comparable PBS/OVA and OVA/OVA groups. In Fig. 9, *P* values of the resulting correlation coefficients were color coded, with red indicating a $P < 0.001$, orange $0.01 > P > 0.001$, and yellow $0.05 > P > 0.01$.

RESULTS

Generation of conditionally Arg1-deficient mice. We flanked exon 4 with *loxP* sites to create a conditionally *Arg1*-deficient mouse. When these $Arg1^{fl/fl}$ mice were crossed with *deleter-Cre* females (48) to delete exon 4 in the germ line, the resulting $Arg1^{-/-}$ mice died between postnatal days 10 and 14, as reported for constitutive *Arg1*-deficient mice (20). At postnatal day 8, plasma levels of arginine and glutamine were approximately nine- and approximately twofold higher, respectively, whereas lysine levels were decreased to ~50% in $Arg1^{-/-}$ (Arg1-KO^{DelCre}) compared with $Arg1^{+/fl}$ (wild-type) mice (Table 2). Although these changes, with the exception of the increased plasma arginine concentration, differ from those reported for *Arg1*-deficient mice in the moribund phase (20), the data show unambiguously that excision of the floxed exon 4 results in a nonfunctional *Arg1* allele.

Arginase expression in the lungs of asthmatic Arg1-Con and Arg1-KO mice. Half of the Arg1-KO^{LysM} or Arg1-KO^{Tie2} mice and their Arg1-Con littermates were sensitized and then challenged with ovalbumin (OVA/OVA). The other 50% of the mice were only challenged with ovalbumin (PBS/OVA). In lungs of OVA/OVA-treated Arg1-Con mice, numerous peribronchiolar, parenchymal, and perivenous arginase 1-positive cells were found, whereas lungs of similarly treated Arg1-KO^{LysM} mice showed fewer peribronchiolar ARG1-positive cells, while no ARG1-positive cells were found in lungs of OVA/OVA-treated Arg1-KO^{Tie2} mice (Fig. 1*D*). Arginase 1 mRNA and protein were hardly detectable in the lungs of mice undergoing the PBS/OVA protocol, whereas a robust increase of arginase 1 mRNA and protein was found in the lungs of OVA/OVA-treated Arg1-Con mice (Figs. 1*B* and 2). The lungs of Arg1-KO^{LysM} OVA/OVA mice contained residual arginase 1 mRNA and protein, but the expression of arginase 1 in the lungs of Arg1-KO^{Tie2} OVA/OVA mice did not exceed that seen in PBS/OVA mice (Figs. 1*B* and 2). Quantification showed that arginase 1 mRNA and protein concentrations in Arg1-KO^{LysM} OVA/OVA lungs were reduced to 40 ± 7 and 32 ± 8%, respectively, of that found in Arg1-Con OVA/OVA mice, and in Arg1-KO^{Tie2} OVA/OVA mice to 4 ± 2 and 2 ± 0.2%, respectively, of that found in Arg1-Con OVA/OVA mice. Of note, we did not observe ARG1-positive endothelial cells in PBS/OVA or OVA/OVA-treated lung arteries (Fig. 1*C*). Since the cell type specificity of the LysM-Cre and Tie2-Cre overlap with respect to *Arg1*-expressing cells, we conclude from these findings that the *Arg1* allele was virtually completely deleted in the lungs of Arg1-KO^{Tie2} mice, including their macrophages, but incompletely in Arg1-KO^{LysM} mice. Since the phenotype of Arg1-KO^{LysM} mice was minimal, we limited the description of our study to Arg1-KO^{Tie2} mice and their Arg1-Con littermates.

Effect of Arg1 ablation on arginine-metabolizing and -transporting genes in lung and on circulating amino acids. We next investigated to what extent *Arg1* ablation caused adaptive changes in the expression of arginine-metabolizing or -transporting proteins (Fig. 2). Compared with PBS/OVA treatment, OVA/OVA induced increases in expression of *Arg1*, *Arg2*, *Nos2*, and *Slc7a2* mRNA in the lungs of Arg1-Con mice, whereas the expression of *Slc7a1* and *Slc7a7* was not changed. In contrast, OVA/OVA treatment failed to induce an increase in expression of *Arg1*, *Arg2*, *Nos2*, and *Slc7a2*, and decreased that of *Slc7a1* and *Slc7a7* in Arg1-KO^{Tie2} mice. Furthermore, we evaluated the expression pattern of arginase 2 protein in sections of PBS/OVA and OVA/OVA-treated Arg1-Con and Arg1-KO^{Tie2} mice (Fig. 3). As expected (59) arginase 2-positive cells with the morphology of macrophages accumulated in the lungs of OVA/OVA-treated mice. Quantification revealed a small increase in the number of ARG2-positive cells after OVA/OVA treatment, compared with PBS/OVA treatment, but no difference was detected between Arg1-Con and Arg1-KO^{Tie2} mice.

Relative to PBS/OVA, OVA/OVA treatment caused a decrease in the concentration of circulating arginine and citrulline (Fig. 2), and asparagine, serine, histidine, glycine, tyrosine, and methionine (Table 3) in Arg1-KO^{Tie2} mice, whereas that of glutamate increased. When OVA/OVA-treated Arg1-Con and Arg1-KO^{Tie2} mice were compared, the latter had lower concentrations of glycine, citrulline, and isoleucine (Table 3).

Arg1 ablation improves peripheral lung function in OVA/OVA-treated mice. To determine whether *Arg1* ablation has an effect on respiratory mechanics, we measured responsiveness

Table 3. *Amino-acid concentrations in venous plasma of Arg1-Con and Arg1-KO^{Tie2} mice*

	Arg1-Con	Arg1-KO^{Tie2}	Arg1-Con	Arg1-KO^{Tie2}
Amino Acid	PBS/OVA	PBS/OVA	OVA/OVA	OVA/OVA
Glu	15 ± 2	13 ± 2	28 ± 1*	26 ± 2†
Asn	39 ± 3	38 ± 3	30 ± 2	26 ± 2†
Ser	136 ± 31	118 ± 22	87 ± 13	52 ± 5†
Gln	834 ± 48	822 ± 25	823 ± 46	752 ± 67
His	110 ± 6	107 ± 5	96 ± 5	84 ± 3†
Gly	297 ± 63	279 ± 30	199 ± 9	155 ± 7†§
Thr	131 ± 6	121 ± 6	125 ± 8	112 ± 5
Cit	75 ± 2	68 ± 3	71 ± 6	57 ± 3§
Arg	102 ± 4	95 ± 5	85 ± 6	76 ± 6†
Ala	750 ± 59	702 ± 31	686 ± 17	604 ± 51
Tyr	174 ± 5	179 ± 11	161 ± 10	134 ± 9†
Tau	189 ± 13	184 ± 13	166 ± 12	144 ± 12
Val	262 ± 13	219 ± 9‡	256 ± 14	218 ± 9
Met	51 ± 2	50 ± 2	46 ± 3	40 ± 2†
Ile	142 ± 9	114 ± 6‡	136 ± 9	111 ± 5§
Phe	32 ± 3	28 ± 2	31 ± 1	25 ± 2
Orn	49 ± 9	34 ± 6	49 ± 5	38 ± 5
Leu	184 ± 11	154 ± 8	178 ± 12	153 ± 7
Trp	235 ± 10	229 ± 10	219 ± 10	214 ± 9
Lys	417 ± 14	397 ± 16	383 ± 21	347 ± 26
Total amino acids	4,233 ± 264	3,959 ± 154	3,865 ± 107	3,379 ± 169

Values are μM ± SE. Arg1-KO^{Tie2}, $Arg1^{fl/fl}/Tie2Cre^{tg/-}$ mice; Arg1-Con, their $Arg1^{fl/fl}/Tie2Cre^{-/-}$ littermates. *$P < 0.05$ PBS/OVA vs. OVA/OVA Arg1-Con; †$P < 0.05$ PBS/OVA vs. OVA/OVA Arg1- KO^{Tie2}; ‡$P < 0.05$ Arg1-Con vs. Arg1-Con^{Tie2} PBS/OVA; §$P < 0.05$ Arg1-Con vs. Arg1-Con^{Tie2} OVA/OVA.

to inhaled methacholine (Fig. 4). Compared with PBS/OVA, OVA/OVA treatment of Arg1-KO^{Tie2} and Arg1-Con mice resulted in an increase in R_N upon exposure to methacholine ($P < 0.04$), albeit without difference between the Arg1-KO^{Tie2} and Arg1-Con mice. *H*, which was higher in OVA/OVA- than in PBS/OVA-treated Arg1-Con mice, was attenuated in OVA/OVA-treated Arg1-KO^{Tie2} mice ($P < 0.0009$). Compared with PBS/OVA, OVA/OVA treatment also increased *G* in Arg1-Con and Arg1-KO^{Tie2} mice upon exposure to methacholine, but the response in Arg1-KO^{Tie2} mice was significantly lower ($P < 0.001$) than in Arg1-Con mice. In aggregate, these findings show that the absence of arginase 1 in the lung does not affect R_N but does affect *H* and *G*, which reflect properties of the lung parenchyma rather than of the larger airways (12, 16).

Arg1 ablation affects levels of mRNA and selected proteins of inflammatory genes in lung. We next investigated whether *Arg1* ablation in the lung had an effect on the expression of asthma-associated cytokines (Fig. 5). As expected, OVA/OVA treatment resulted in an increased abundance of *Il4*, *Il13*, *Il5*, *Ccl11*, *Ccl2*, and *Il10* mRNAs in Arg1-Con mice. The OVA/OVA-induced increase in *Ccl11* expression was markedly reduced in Arg1-KO^{Tie2} mice. Furthermore, the expression of the inflammatory markers *Tnfa* and *Ifng* was strongly suppressed. The expression of the respiratory epithelium-specific *Muc5ac* and *Clca3* genes was increased upon OVA/OVA treatment in both Arg1-Con and Arg1-KO animals, but the response was markedly attenuated in Arg1-KO^{Tie2} mice, in particular with respect to *Clca3*.

To investigate whether *Arg1* deletion in the lung also affected the pulmonary protein concentration of cytokines and chemokines, we performed ELISAs for IL-4, IL-13, IL-5, CCL11 (eotaxin-1), IL-10, TNF-α, and IFN-γ on whole lung extracts (Fig. 6). Except for IL-10 and IFN-γ, the pulmonary concentration of these proteins increased after OVA/OVA treatment, but the increase in IL-13 and CCL11 concentration was significantly lower in Arg1-KO^{Tie2} than in Arg1-Con mice. These data show that the production of most asthma-associated cytokines proceeded independently of *Arg1* deficiency in the lung.

Effect of Arg1 deletion on the production of OVA-specific IgE. OVA-specific IgE was undetectable in plasma of PBS/OVA-treated mice but increased to easily detectable concentrations after OVA/OVA treatment in both wild-type and knockout mice, without significant differences between animals with or without *Arg1* ablation (Fig. 6). Apparently, ablation of *Arg1* in Arg1-KO^{Tie2} mice did not affect the adaptive immune response to ovalbumin.

Effects of Arg1 deletion on tissue inflammation. Finally, we investigated whether *Arg1* ablation in the lung modified the allergic asthma-induced inflammatory response of the lung (Fig. 7). We did not observe any inflammatory cells in H&E- or ARG1-stained lung sections of PBS/OVA-treated mice (Fig. 7*A*, *top row*, and Fig. 1*D*). Furthermore, there were no cells that contained MBP and MPO, which are markers for eosinophils and neutrophils, respectively (Fig. 7*A*, *middle* and *bottom rows*). In lungs of OVA/OVA-treated mice, on the other hand, many inflammatory cells were present, without discernible differences between Arg1-KO^{Tie2} mice and their Arg1-Con littermates. In lung sections stained for Sirius red and reticulin we found no differences between the OVA/OVA-treated Arg1-Con and Arg1-KO^{Tie2} mice in the amount of stained fibers (Fig. 8). We quantified the number of MBP- and MPO-positive cells, and did not find differences between Arg1-KO^{Tie2} mice and their Arg1-Con littermates either (Fig. 7*B*). Furthermore, lung sections stained for the presence of collagen and reticular fibers showed a similar degree of remodeling upon OVA/OVA treatment between Arg1-Con and Arg1-KO^{Tie2} mice (Fig. 8). Apparently, *Arg1* ablation in the lung does not affect the infiltration of inflammatory cells or tissue remodeling in response to experimental asthma, nor does it induce or reduce remodeling effects.

Comparison of the adaptive responses of Arg1-Con and Arg1-KO^{Tie2} mice. To answer the question whether arginase 1 activity exerted a coherent influence on the adaptive responses to asthma, we also investigated the correlation coefficients between each of the lung-function parameters, mRNA and protein concentrations, histology scores and plasma amino-acid concentrations (Fig. 9). In Arg1-Con mice, lung function parameters *H* and *G* corresponded well mutually and with IL-5, IL-13, and CCL11. Except for *Slc7a7*, *Slc7a1*, *Tnfa*, and *Ifng*

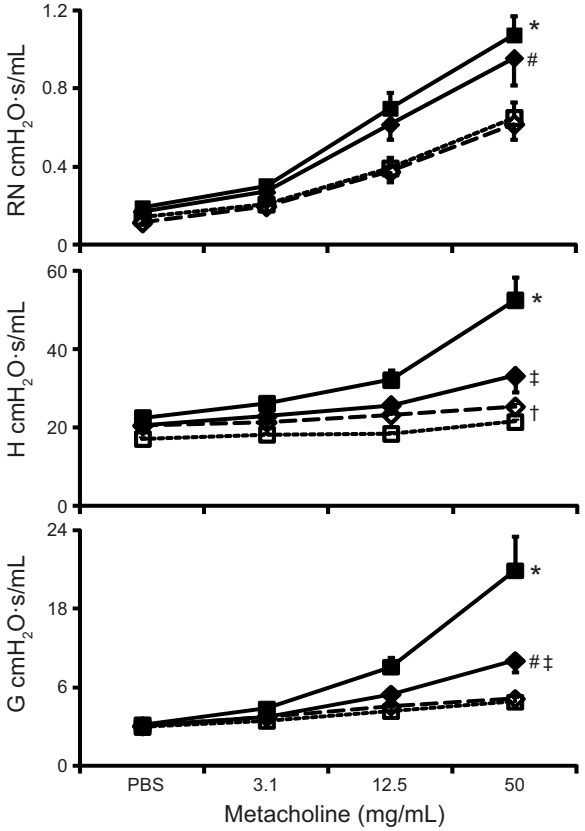

Fig. 4. Lung function in mouse lungs that are or are not *Arg1* deficient. The responsiveness to increasing doses of aerosolized methacholine on airway resistance (R_N), tissue elastance (*H*), and tissue resistance (*G*) was measured. PBS/OVA-treated Arg1-Con mice: □ and dotted line; PBS/OVA-treated Arg1-KO^{Tie2} mice: ◇ and dashed line. OVA/OVA-treated Arg1-Con mice: ■ and solid line; OVA/OVA-treated Arg1-KO^{Tie2} mice: ◆ and solid line. Values on the *x*-axis represent the aerosolized methacholine concentrations used (mg/ml). Mean ± SE (PBS/OVA-treated Arg1-Con: $n = 7$; Arg1-KO^{Tie2}: $n = 8$; OVA/OVA-treated Arg1-Con: $n = 10$, Arg1-KO^{Tie2}: $n = 9$). *$P < 0.05$ OVA/OVA vs. PBS/OVA Arg1-Con^{Tie2}; #$P < 0.05$ OVA/OVA vs. PBS/OVA Arg1-KO^{Tie2}; ‡$P < 0.01$ OVA/OVA Arg1-KO^{Tie2} vs. Arg1-Con^{Tie2}; †$P < 0.001$ PBS/OVA Arg1-KO^{Tie2} vs. Arg1-Con^{Tie2}.

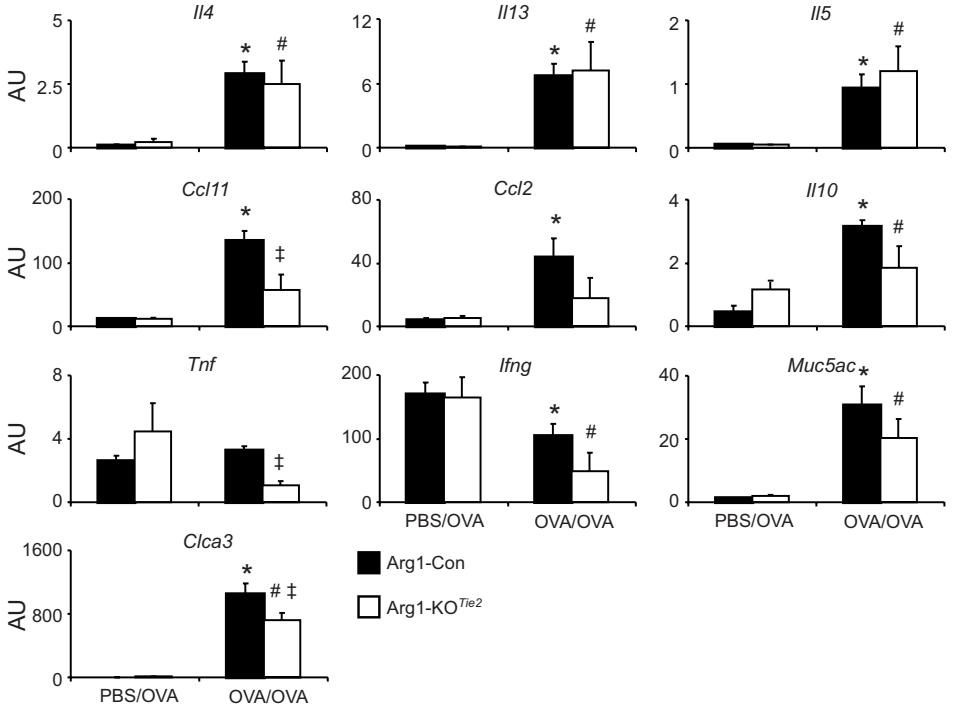

Fig. 5. Inflammatory gene expression in mouse lungs that are or are not *Arg1* deficient. Arg1-Con and Arg1-KO^{Tie2} are represented by solid and open bars, respectively. Treatment (PBS/OVA or OVA/OVA) is indicated below the bars. Pulmonary expression of T_H2-related inflammatory genes *Il4*, *Il13*, *Il5*, and *Ccl11*, macrophage-chemotactic protein *Ccl2*, anti-inflammatory gene *Il10*, T_H1-related inflammatory genes *Ifng* and *Tnfa*, and marker genes for the activation of bronchiolar epithelium *Muc5ac* and *Clca3* is shown as means ± SE (PBS/OVA-treated Arg1-Con: $n = 7$; Arg1-KO^{Tie2}: $n = 8$; OVA/OVA-treated Arg1-Con: $n = 10$; Arg1-KO^{Tie2}: $n = 9$). The number of mRNA copies was normalized for 18S rRNA expression and multiplied by 10,000. *$P <$ 0.05 PBS/OVA vs. OVA/OVA Arg1-Con; #$P < 0.05$ PBS/OVA vs. OVA/OVA Arg1-KO^{Tie2}; ‡$P < 0.05$ OVA/OVA Arg1-KO^{Tie2} vs. Arg1-Con.

mRNAs, mRNA concentrations correlated very well mutually and with the corresponding proteins (including ARG1). Tissue concentrations of IL-4, IL-13, IL-5, CCL11, and TNF-α correlated mutually. Finally, it should be mentioned that IL-10, IFN-γ, and OVA-specific IgE did not correlate tightly with any of the other markers investigated. In aggregate, these data show that *H*, *Slc7a2b*, *Arg1*, *Nos2*, *Il4*, *Il13*, *Il5*, *Ccl11*, *Il10*, *Ccl2*, *Muc5ac*, and *Clc3a* mRNA, ARG1, IL-4, IL-13, IL-5, CCL11, and TNF-α protein are sensitive parameters to assess quantitative differences in the allergic pulmonary response to OVA. The best overall cross-correspondence was seen for *H*, *Il4*, *Il13*, and *Il5* mRNA, IL-13, IL-5, CCL11, and TNF-α protein. Striking were the weak cross-correspondences of these parameters with R_N and mRNAs coding for arginine-metabolizing and transporting proteins, Furthermore, only the density of MBP-positive cells in the lung correlated weakly with lung function and gene expression, whereas virtually no correlation was found for MPO-positive cells.

Relative to Arg1-Con mice, Arg1-KO^{Tie2} mice showed a pronounced general loss of correspondence of OVA/OVA-sensitive parameters (Fig. 9). A few correlations became stronger, such as that of *H* with *Nos2* mRNA, IL-4 protein, and plasma citrulline concentrations and that of *Arg1* mRNA with IL-4, IL-5, CCL11, and TNF-α protein concentrations. In aggregate, the data show that the near complete elimination of *Arg1* expression from the lung disturbed the coordinated pulmonary response to OVA/OVA treatment, that is, showed that some aspect of arginine metabolism coordinated this response.

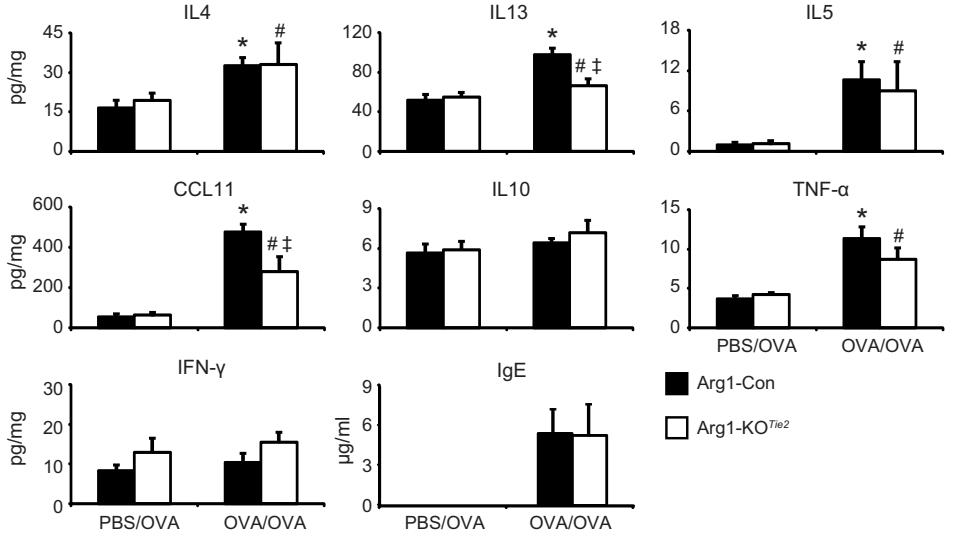

Fig. 6. Pulmonary concentration of inflammatory cytokines, chemokines, and OVA-specific IgE in mouse lungs that are or are not *Arg1* deficient. Arg1-Con (solid bars) and Arg1-KO^{Tie2} (open bars) mice. Treatment of the mice (PBS/OVA or OVA/OVA) is indicated. The pulmonary concentration of IL-4, IL-13, IL-5, CCL11, IL-10, TNF-α, and IFN-γ, and OVA-specific IgE in plasma is shown as means ± SE (PBS/OVA-treated Arg1-Con: $n = 7$; Arg1-KO^{Tie2}: $n = 8$; OVA/OVA-treated Arg1-Con: $n = 10$; Arg1-KO^{Tie2}: $n = 9$). *$P < 0.01$ vs. PBS/OVA Arg1-Con; #$P < 0.01$ vs. PBS/OVA Arg1-KO$^{LysM/Tie2}$; ‡$P < 0.001$ vs. OVA/OVA Arg1-Con.

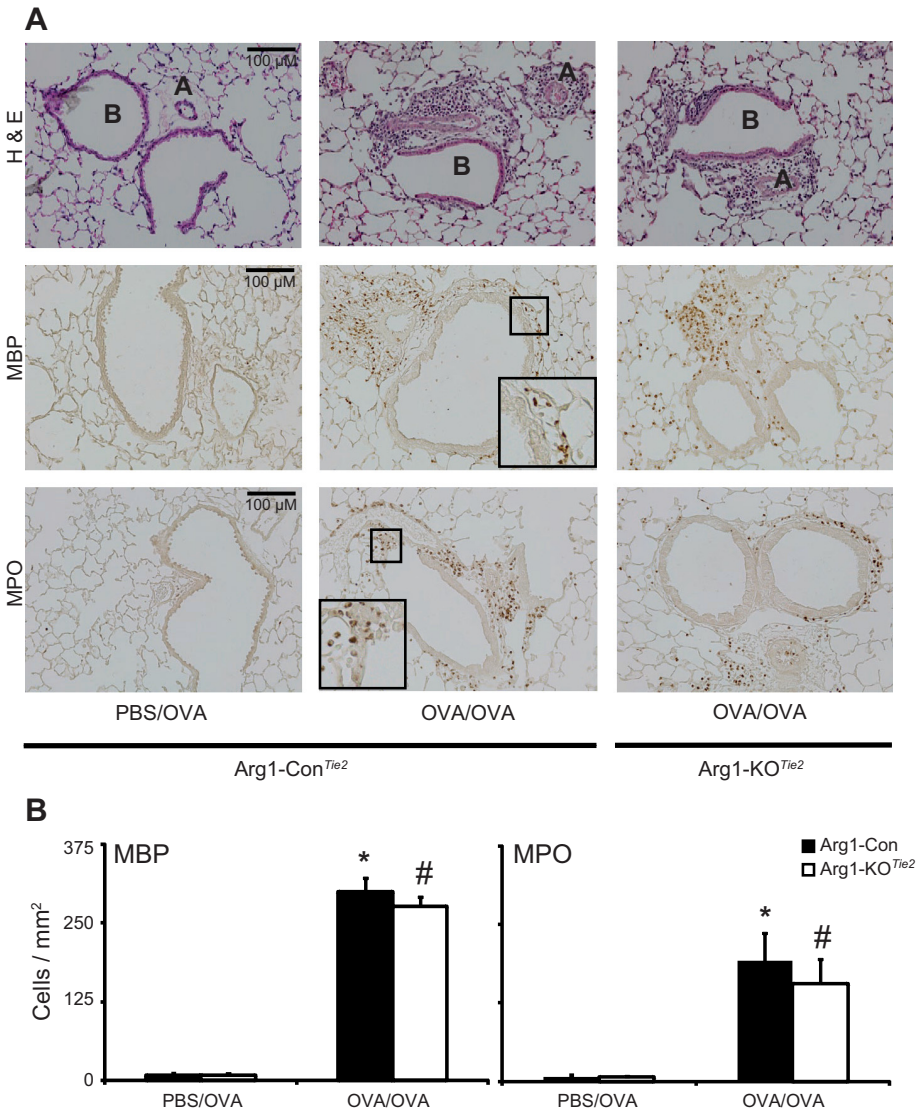

Fig. 7. Prevalence of inflammatory cells in mouse lungs that are or are not *Arg1* deficient. *A*: lung sections. *Left* 2 bars: PBS/OVA- and OVA/OVA-treated Arg1-Con mice. *Right* bar: OVA/OVA-treated Arg1-KO^{Tie2} mice. *Top row*: H&E stain. *Middle row*: MBP stain (identifies eosinophilic cells). *Bottom row*: MPO stain (identifies neutrophils). Bar: 100 μm. *B*: quantification of inflammatory cells in PBS/OVA or OVA/OVA-treated Arg1-Con (solid bars) and Arg1-KO^{Tie2} (open bars) mice. Sections were stained simultaneously. Values are shown as means ± SE (PBS/OVA-treated Arg1-Con: n = 7; Arg1-KO^{Tie2}: n = 8; OVA/OVA-treated Arg1-Con: n = 10; Arg1-KO^{Tie2}: n = 9). *P < 0.01 vs. PBS/OVA-treated Arg1-Con; #P < 0.01 vs. PBS/OVA-treated Arg1-KO^{Tie2}.

DISCUSSION

We generated mice in which exon 4 of the *Arg1* gene was floxed and effectively ablated arginase 1 expression in the lung by crossing them with Tie2Cre mice. Ablation of *Arg1* in hematopoietic and endothelial cells did not affect OVA sensitization, as reflected in a similar increase in OVA-specific IgE in plasma of Arg1-Con and Arg1-KO mice. OVA sensitization and challenge of Arg1-KO mice did not affect R_N but attenuated *H* and *G*, indicating that *Arg1* ablation mainly affected the lung parenchyma. Furthermore, it reduced the mRNA abundance of *Arg2*, *Slc7a1*, *Slc7a7*, *Ccl11*, *Tnfa* and *Clca3* in OVA/OVA-treated mice. Correlation analysis revealed that *Arg1* ablation in the lung disturbed the coordinated pulmonary response to OVA/OVA treatment and made it more dependent on adaptive changes in arginine metabolism.

All our experiments were carried out in mice constructed in the 129Sv strain and backcrossed to the C57BL/6J strain. Since allergic asthma is considered a T_H2-dominated immune disorder, it is sometimes argued that the T_H1-responsive C57BL/6J mouse strain is less suitable to OVA sensitization and challenges than e.g., the T_H2-responsive BALB/c mouse strain. However, (nearly) all genetically modified mice are bred in the C57BL/6J strain. To avoid undue influence of the genetic background of the mice, we used *Arg1$^{fl/fl}$/Tie2Cre$^{-/-}$* littermates of *Arg1$^{fl/fl}$/Tie2Cre$^{tg/-}$* mice as reference throughout the study.

Macrophages are the main myeloid-derived cells that express arginase upon induction by T_H2 cytokines in rodents (37). Because no macrophage-selective Cre line exists, we used both LysMCre (9) and Tie2Cre (23) mice to bring about *Arg1* ablation in macrophages. Whereas the expression of LysMCre is confined to macrophages and granulocytes (9), that of Tie2Cre includes all hematopoietic and endothelial cells (47). However, we found that the endothelium of lung arteries does not contain arginase 1, whereas, to the best of our knowledge, murine lymphocytes also do not express arginase. Furthermore, Tie2Cre-mediated ablation is usually complete (15, 23, 25, 47), whereas LysMCre-mediated ablation is notably less effective, with target excision in 50–80% of the target cells (9, 14, 21, 58). In agreement with these earlier findings, we observed a much smaller reduction of arginase 1 protein in the lungs of OVA/OVA-treated Arg1-KO^{LysM} mice (to

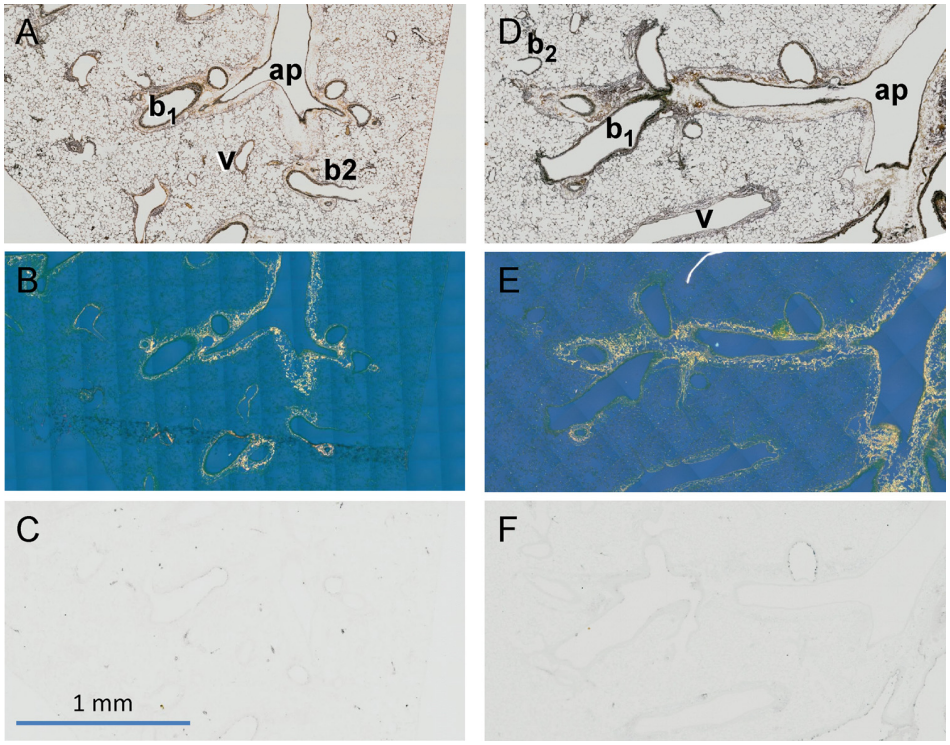

Fig. 8. Lung sections of OVA/OVA-treated mice stained for the presence of reticular and collagen fibers, and NOS2. Serial sections of Arg1-KO (*A–C*) and Arg1-Con mice (*D–F*) were stained with silver nitrate (reticular fibers; *A* and *D*), Sirius red (collagen fibers; *B* and *E*), or NOS2 (*C* and *F*). Note presence of additional reticular and collagen fibers in the cuff around main bronchi (b1) and arteries of OVA/OVA-treated mice, but the absence of such fibers around these structures in the parenchyma (b2). NOS2 was only found in the epithelium of bronchi that were not enveloped in connective-tissue sheaths. ap, Pulmonary artery; v, pulmonary vein.

~30%) than in the lungs of similarly treated Arg1-KO^{Tie2} mice or in thioglycollate-elicited peritoneal macrophages of these mice (53) (to ~2%). Of interest, a recent study (9a) identified the ARG1-positive macrophages as CD11b$^+$Gr1$^+$F4/80$^+$Ly-6C$^-$Ly-6G$^+$ myeloid-derived suppressor cells and ascribed to them, together with the CD11b$^+$Gr1$^+$F4/80$^+$Ly-6C$^-$Ly-6G$^+$ monocytoid cells, which mainly produce NO, a key coordinating role in the manifestation of allergic asthma. The expression of arginase 1 in cells that resemble lung macrophages and the near-complete elimination of *Arg1* expression from the lung and from peritoneal macrophages in Arg1-KO^{Tie2} mice, therefore, shows that we did target arginase 1 expression in macrophages and that the present study allows conclusions with respect to the role of arginase 1 in allergic asthma of mice. Since the described phenotype was only observed in the Tie2 line, *Arg1* deletion in macrophages apparently had to be near complete to mediate pronounced effects.

Arg1 ablation affects the expression of genes coding for arginine-metabolizing and -transporting proteins. The genetic elimination of *Arg1* in macrophages of OVA/OVA-treated mice decreased the expression of *Arg2*, *Slc7a1* (*Cat1*), and *Slc7a7* (*Lat1*) mRNAs. These effects point to an arginase 1-dependent, and, hence, probably an arginine concentration-dependent decline in expression of genes involved in arginine transport and metabolism. The OVA/OVA-mediated increase in pulmonary arginase activity is reported to reduce parenchymal arginine content approximately twofold (22), but did not prevent an approximately twofold increase in NO production (4).

Effect of Arg1 ablation on lung mechanics. R_N in the large airways of OVA/OVA-treated *Arg1*-deficient mice was not different from that in similarly treated animals without the deletion, but *H* and *G* were markedly lower in the Arg-KO^{Tie2} mice. *H* and *G* primarily reflect peripheral lung function (1) and may, in allergic asthma, reflect "closure" of peripheral airways by mucous bridges (30, 31, 55). In agreement, the mRNA abundance of *Clca3* (*Gob5*), which regulates mucus production, and the mucin *Muc5ac* were lower in OVA/OVA-treated Arg1-KO^{Tie2} than Arg1-Con mice. Overexpression of *Clca3* exacerbates AHR in OVA/OVA-treated mice, whereas downregulation attenuates peak airway pressure (resistance) after methacholine (29, 38), strongly suggesting a role for this gene in the development of peripheral responsiveness. On the basis of our observations in sections stained for the presence of collagen and reticular fibers, the improved peripheral lung function could not be caused by less remodeling in OVA/OVA-treated Arg1-KO^{Tie2} mice compared with Arg1-Con mice.

The effects of arginase 1 activity on the biomechanics of asthmatic lungs were also studied pharmacologically. Inhibitors of arginase activity decreased airway resistance in OVA/OVA-treated guinea pigs (33, 34) and mice, but did not change (19, 40) or even increased (7) *H* and *G* in mice. These pharmacological findings differ from the present findings in a genetic model and suggest that systemic and, in our case, macrophage-specific suppression of arginase activity has different effects. In fact, the presence or absence of arginase 1 in lung macrophages seems to be more important than its concentration, because the strong increase in *Arg1* and *Arg2* expression that was observed in OVA/OVA-treated *Nos2*-deficient mice compared with control (C57BL/6) mice (4) did not affect AHR (and eosinophil influx) (42). On the basis of our findings in an OVA/OVA-treated arginine-deficient mouse model (our unpublished observations), we speculate that changes in the circulating concentration of arginine primarily affect the function of the larger airways, whereas the local presence or absence of arginase 1 primarily affects the function of the peripheral airways. This explanation

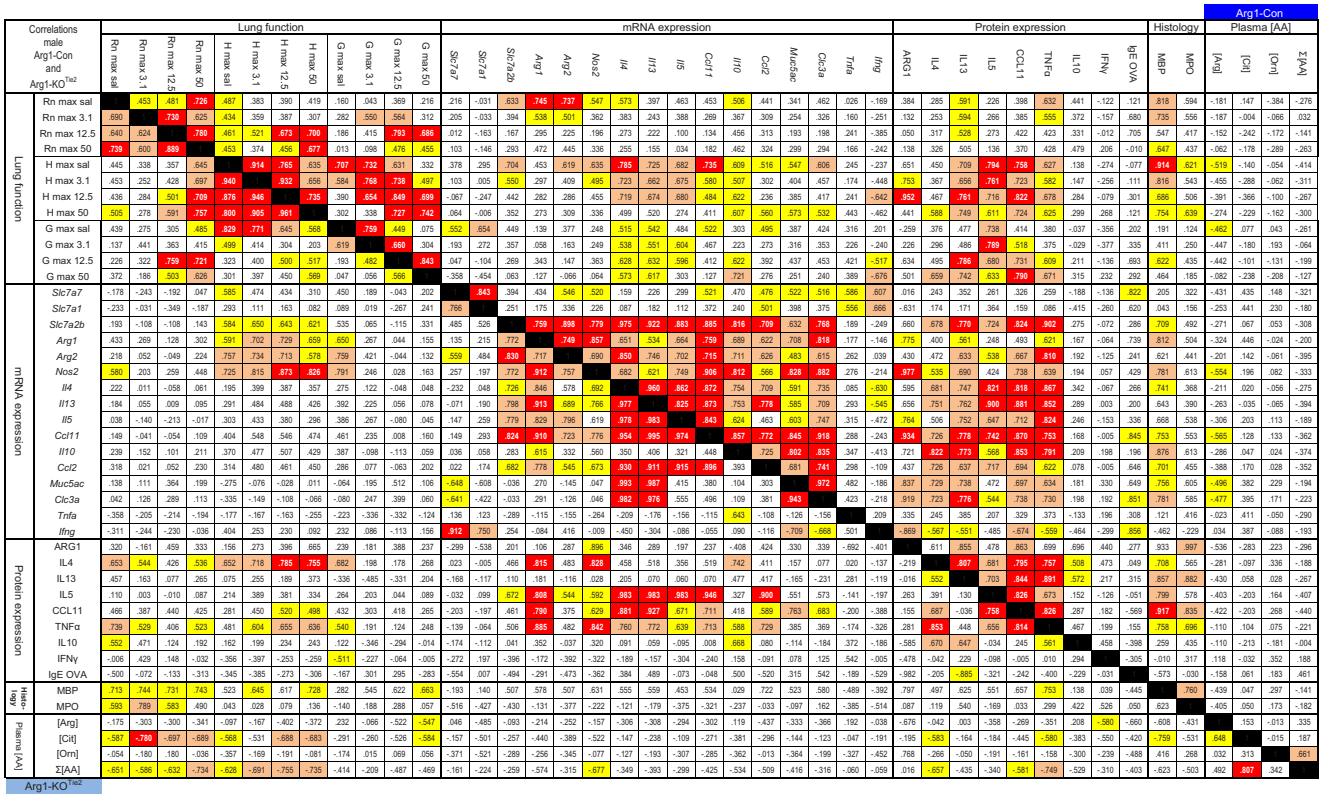

Fig. 9. Correlation of lung function, abundance of pulmonary mRNAs and proteins, pulmonary histopathology, and concentrations of plasma amino acids in mice with lungs that are or are not *Arg1* deficient. *Top right* triangle refers to data obtained in Arg1-Con mice and *bottom left* triangle to data from Arg1-KO^{Tie2} mice. Numbers show the correlation coefficients between parameters indicated above and to the left of the columns and rows, respectively, as measured in all 15 or 16 mice of the Arg1-Con or Arg1-KO^{Tie2} genotype studied, that is, both the PBS/OVA- and the OVA/OVA-treated groups. The significance of the correlations is color coded according to the P value of the correlation coefficient: yellow $0.05 > P > 0.01$, orange $0.01 > P > 0.001$, and red $P < 0.001$. Note the decline of the number or degree of significant correlations in Arg1-KO^{Tie2} compared with Arg1-Con mice, in particular with respect to lung function and abundance of pulmonary mRNAs and proteins.

would accommodate the observed differences between the pharmacological and genetic interventions.

Effect of Arg1 ablation in the lung on pulmonary inflammation. The allergic response to OVA challenge is at least partly mediated by OVA-specific IgE (49). Unexpectedly, therefore, circulating anti-OVA IgE concentrations did not correlate with any other parameter of allergic asthma (Fig. 9). OVA/OVA treatment resulted in a pan-pulmonary cellular inflammation in all mice, with participation of both eosinophils (MBP) and neutrophils (MPO). The relatively low number neutrophils relative to eosinophils in the lung of OVA/OVA-treated mice can be ascribed to our treatment protocol, as neutrophils preferentially infiltrate the lung shortly after a challenge (<24 h) and eosinophils later (43). For the same reason, our relatively extended challenge protocol (6 consecutive days) probably accounts for the observation that only the number of eosinophils corresponded with the functional and biochemical sequels of OVA/OVA treatment (Fig. 9). One or more of the inflammatory cell types produced and induced an increased expression of mRNAs of cytokines and chemokines that are involved in T_H2 responses (*Il4*, *Il13*, *Il5*, *Ccl11*), macrophage function (*Ccl2*, *Il10*), and mucus production in the respiratory epithelium (*Muc5ac*, *Clca3*). The expression of *Muc5ac* and *Clca3* correlated well with the expression of genes coding for arginine-metabolizing and -transporting proteins in Arg1-Con mice, but this coordination was largely lost in Arg1-KO^{Tie2} mice, implying that cellular arginine concentration plays a role in their expression. In contrast, the abundance of *Il4*, *Il13*, *Il5*, *Ccl2*, and *Ccl11* mRNA remained coordinated irrespective of the presence or absence of *Arg1*, implying no or a limited role of arginase 1. *Arg1* deficiency in OVA/OVA-treated mice led to reduced *Ccl11*, *Tnfa* and *Clca3* expression and CCL11 protein accumulation. The lower CCL11 (eotaxin-1) content, however, did not result in the recruitment (13) of fewer eosinophils to the lungs. Furthermore, the pulmonary content of IL-13, a cytokine associated with AHR induction (35), was reduced.

Posttranscriptional regulation of genes in OVA/OVA-treated mice. For arginase 1, IL-4, IL-13, IL-5, IL-10, CCL11, TNF-α, and IFN-γ, we measured both mRNA and protein concentrations in lung homogenates. Correlations varying from reasonable ($P < 0.05$) to good ($P < 0.01$) were found between mRNA and protein levels in Arg1-Con animals for IL-4, IL-13, IL-5, and CCL11 (Fig. 9), but in Arg1-KO^{Tie2} mice, only mRNA and protein concentrations of IL-5 and CCL11 still correlated. Posttranscriptional control is well established for the expression of cytokines (8), but we were not aware of such extensive posttranscriptional regulation of *Arg1* expression. Because we wanted to study the coordinated pulmonary response to ovalbumin sensitization and challenges in the pres-

ence or absence of arginase 1 enzyme activity, we have only determined changes in mRNA levels.

Comparison with other animal models with macrophage-specific Arg1 deletion. Recently, the effects of macrophage-specific *Arg1* deficiency on lung function and inflammation were studied in OVA/OVA-treated wild-type mice after bone marrow transplantation with constitutively *Arg1*-deficient cells (39), a procedure that also involves *Arg1* elimination in all bone marrow-derived cells. Comparable to our results, infiltration of the lungs with eosinophils and neutrophils was similar in mice with or without *Arg1* ablation in hematopoietic cells. Lung function was assessed with the airway pressure-time index, which is less sensitive to changes in peripheral lung function (45) and may explain why no differences in lung function were found in that study (39).

The effects of arginase 1 activity on the biomechanics of asthmatic lungs were also studied with RNA interference and administration of arginase inhibitors. Inhibition of *Arg1* expression by instilling anti-*Arg1* shRNA in the trachea of mice attenuated IL-13-induced airway resistance (Raw) (57), whereas we found that genetic ablation did not have this effect. The discrepancy may be caused by the amount of rIL13 instilled into the lung (10 μg), which exceeds the content of IL-13 in OVA/OVA-treated lungs (~100 pg/mg lung protein) by three orders of magnitude. Pharmacological inhibition of arginase activity decreased R_N in OVA/OVA-treated guinea pigs (32, 34) and mice (19, 40) but did not change *H* or *G*, although one mouse study found no change R_N and an increase in *H* and *G* (7).

Both *Nos1*- and *Nos3*-deficient mice suffer from a more pronounced AHR than wild-type mice (17, 18), whereas in *Nos3*-overexpressing mice, allergen-induced AHR to methacholine is completely abolished (11, 51), indicating a prime role for NO in airway relaxation. In agreement, inhibition of arginase activity in OVA/OVA-treated *Nos2*$^{-/-}$ mice did not induce AHR, suggesting that NOS1 and -3 used the increased tissue arginine levels to produce more NO and relax the smooth muscles of the larger airways (42). Similarly, inhibition of NOS2 almost completely abolished OVA/OVA-induced AHR in BALB/c mice (24). Since *Nos2* overexpression also decreases airway resistance (Raw) (17a), the source of NO is apparently less important.

The use of strong arginase inhibitors like nor-NOHA increase tissue arginine levels and corresponding decreases in AHR and eosinophil influx in C57BL/6 mice (5, 39). In another series of experiments with arginine- rather than *Arg1*-deficient mice (our unpublished observations), we found that circulating arginine concentrations primarily affected R_N in the larger airways. We, therefore, hypothesize that ablation of *Arg1* in lung macrophages increases the parenchymal arginine concentration, which, in turn, facilitates NO production and smooth-muscle relaxation in the peripheral airways, but not the larger conducting airways.

Conclusion. Ablation of *Arg1* in macrophages leads to an improvement of peripheral lung function in OVA/OVA-treated Arg1-KO^{Tie2} mice, without altering air hyperreactivity and lung inflammation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

R.H.E.C., S.S., C.C.d.T., M.E.P., E.T., P.v.D., and T.B.M.H. performed experiments; R.H.E.C., S.S., and S.E.K. analyzed data; R.H.E.C., S.S., and W.H.L. interpreted results of experiments; R.H.E.C. and S.E.K. prepared figures; R.H.E.C. and S.S. drafted manuscript; R.H.E.C., S.S., C.C.d.T., M.E.P., E.T., P.v.D., T.B.M.H., W.H.L., and S.E.K. approved final version of manuscript; C.C.d.T., M.E.P., W.H.L., and S.E.K. edited and revised manuscript; W.H.L. and S.E.K. conception and design of research.

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CHAPTER IV

THE HUMAN NEONATAL SMALL INTESTINE HAS THEPOTENTIAL FOR ARGININE SYNTHESIS; DEVELOPMENTAL CHANGES IN THE EXPRESSION OF ARGININE–SYNTHESIZING AND –CATABOLIZING ENZYMES

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The human neonatal small intestine has the potential for arginine synthesis; developmental changes in the expression of arginine-synthesizing and -catabolizing enzymes

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Abstract

Background: Milk contains too little arginine for normal growth, but its precursors proline and glutamine are abundant; the small intestine of rodents and piglets produces arginine from proline during the suckling period; and parenterally fed premature human neonates frequently suffer from hypoargininemia. These findings raise the question whether the neonatal human small intestine also expresses the enzymes that enable the synthesis of arginine from proline and/or glutamine. *Carbamoylphosphate synthetase (CPS), ornithine aminotransferase (OAT), argininosuccinate synthetase (ASS), arginase-1 (ARG1), arginase-2 (ARG2), and nitric-oxide synthase (NOS) were visualized by semiquantitative immunohistochemistry in 89 small-intestinal specimens.*

Results: Between 23 weeks of gestation and 3 years after birth, CPS- and ASS-protein content in enterocytes was high and then declined to reach adult levels at 5 years. OAT levels declined more gradually, whereas ARG-1 was not expressed. ARG-2 expression increased neonatally to adult levels. Neurons in the enteric plexus strongly expressed ASS, OAT, NOS1 and ARG2, while varicose nerve fibers in the circular layer of the muscularis propria stained for ASS and NOS1 only. The endothelium of small arterioles expressed ASS and NOS3, while their smooth-muscle layer expressed OAT and ARG2.

Conclusion: The human small intestine acquires the potential to produce arginine well before fetuses become viable outside the uterus. The perinatal human intestine therefore resembles that of rodents and pigs. Enteral ASS behaves as a typical suckling enzyme because its expression all but disappears in the putative weaning period of human infants.

Background

Arginine is a precursor for the synthesis of proteins, creatine, agmatine, and nitric oxide (NO). It further plays an essential role in ammonia and bicarbonate detoxification, and stimulates the secretion of growth hormone, prolactin, insulin, and glucagon. Arginine is also a 'conditionally essential' amino acid, meaning that endogenous arginine production covers metabolic requirements in healthy, unstressed individuals, but becomes an essential amino acid under conditions of increased need, e.g. growth or tissue repair, or in catabolic states such as sepsis and starvation.

In the adult, endogenous arginine biosynthesis is an inter-organ 'affair': the net production of citrulline occurs almost exclusively in the enterocytes of the small intestine [1], also in man [2], but absorption of citrulline from the circulation and subsequent biosynthesis of arginine can take place in many tissues [3]. Of these, the cortex of the kidney provides approximately 20% of whole-body requirements [4]. In perinatal mice [5,6] and piglets [7-9], however, all enzymes necessary for arginine biosynthesis from proline and glutamine (Figure 1) are expressed in the enterocytes of the small intestine, while ARG1, the main cytosolic arginine-catabolizing enzyme, is not detectable prior to weaning [5,6,10]. In agreement, the small intestine plays a prominent role in net arginine production in suckling piglets [11-14]. In rodents, intestinal expression of the enzymes that synthesize arginine from citrulline, ASS and argininosuccinate lyase, ceases completely after weaning [6,15]. In pigs, on the other hand, net synthesis of arginine declines more gradually and is still present at 7 weeks of age [16]. It has been speculated that enteric arginine synthesis is necessary to cover neonatal requirements, because mammalian milk is a relatively poor source of arginine, whereas its precursors proline and glutamine are abundant [17].

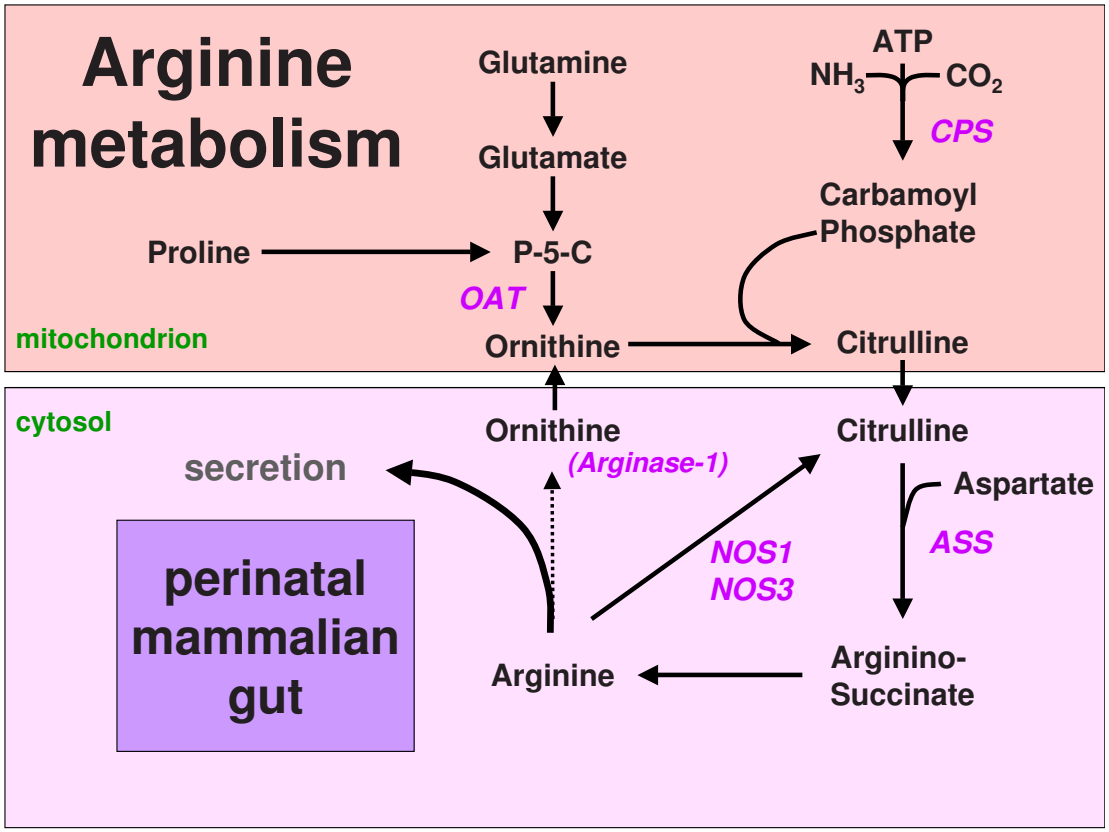

Figure 1
Arginine synthesis from proline or glutamine in the mammalian neonatal gut. Since arginase-1 is not expressed, arginine can either be secreted or metabolized to NO and citrulline. Names of enzymes investigated in this study are indicated in italics. P-5-C = pyrroline5-carboxylate synthetase.

In prematurely born human neonates, hypoargininemia is frequently observed [18] and hypothesized to predispose such infants to the development of necrotizing enterocolitis [19-21]. Although hypoargininemia in premature human neonates has been associated with failing intestinal arginine biosynthesis as found in suckling rodents and piglets [22], no evidence to support this association exists thus far. To confirm or reject the hypothesis that the perinatal human gastrointestinal tract resembles that of rodents or pigs with respect to arginine production, we studied the developmental changes in the expression of CPS, OAT and ASS, three key enzymes with a high control of de novo intestinal synthesis of citrulline and arginine, and ARG1 and ARG2, the main arginine-catabolizing enzymes in full-thickness and mucosal biopsies of the human small intestine. The findings demonstrate that the epithelium of the fetal and neonatal small intestine abundantly expresses CPS (as we reported earlier [23,24]), OAT and ASS, whereas cytosolic ARG1 is not detectable. These data show that the perinatal human intestine resembles that of rodents and, in particular, pigs with respect to its capacity to produce arginine. We also show that the expression of the controlling enzyme, ASS, all but disappears between 3 and 5 years of age, that is, the putative weaning age of human infants [25]. Finally, we show that the enteric ganglia and arteriolar endothelium co-express ASS and the constitutive NO-synthases NOS1 and NOS3, respectively, which both use arginine as substrate for NO synthesis.

Methods

Tissue

A total of 89 samples were included in the study (Table 1). Formalin-fixed, paraffin-embedded samples originated from the archives of the Institute of Pathology, University Hospital Basel, Switzerland, and the Department of Pathology, AMC, Amsterdam, the Netherlands. Full-thickness duodenal, jejunal and ileal samples were from infants, who presented with gastroschisis, atresia, meconium ileus or Meckel's diverticulum, whereas the samples from adults were from patients who underwent surgery for tumors. Duodenal mucosal biopsies were collected from patients who underwent endoscopy for various gastrointestinal complaints. Furthermore, full-thickness intestinal samples of 9 fetuses between 14 and 40 gestational weeks were examined. For each sample, age, gender and diagnosis were available, but in all other respects, the samples had been anonymized to avoid patient identification. Under this condition, residual tissue could be used for the research reported here [26]. In addition, approval has been obtained from the Ethikkommission beider Basel EKbB, Switzerland, reference number EK 135/08 for the samples obtained from the Institute of Pathology in Basel.

Immunohistochemistry

Tissue was fixed in 4% formaldehyde and embedded in paraffin following standard protocols. 5–7 μm-thick sections were used. Section thickness does not affect immunohistological staining intensity, since antibodies only bind to the surface of the sections [27]. As an additional cautionary measure to avoid staining differences due to differences in fixation, we used neuronal staining as an internal reference. Sections were deparaffinized, hydrated in graded ethanols, and heated in 10 mM sodium citrate (pH 6.0) to 98°C for 10 minutes followed by 90 minutes cool-down to room-temperature to retrieve antigens. After this treatment, endogenous alkaline phosphatases are denatured and no longer active. Remaining activity of endogenous peroxidases (catalase) was inactivated where appropriate by exposing the sections for 30 min to 3% H_2O_2 in PBS. After blocking with TENG-T (10 mM Tris pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20) plus 10% goat serum, sections were incubated overnight at room temperature with the first antibody, washed in 0.5 M Na-acetate and incubated with an alkaline phosphatase- or peroxidase-coupled secondary antibody for 60 to 90 minutes. Sections were developed with

Table 1: Human gut samples

Group	Age	# of samples	Sample origin				Resections	Biopsies
			SI	*D*	*J*	*I*		
1	-182/-3 days	9	7		2		9	0
2	1 – 11 days	14	1	4	3	6	14	0
3	6 wks – 1 yr	10	4	1		5	9	1
4	1,5 – 3 yrs	12	2	7		3	5	7
5	3 – 5 yrs	16*		15		3	0	16
6	5 – 7 yrs	18*		16		5	2	16
7	14 – 22 yrs	5		1		4	4	1
8	50 – 80 yrs	5		4		1	5	0

The table shows the number of samples that were analyzed per age group, the topographic location of the samples (SI = small intestine, D = duodenum, J = jejunum, I = ileum), and whether they were mucosal biopsies and full-thickness resections. For age groups 5 and 6, parallel samples were available from two different regions of the small intestine. These were used to check for the effect of location on enzyme expression, but only one sample per patient was taken into account for the statistical analysis. This is the reason for the discrepancy between "# of samples" (marked by an asterisk) and the sum of all samples listed under "Sample origin".

NBT/BCIP (alkaline phosphatase; Roche) or DAB (peroxidase; Sigma). An incubation without primary antibody served as negative control for all incubations [see Additional file 1]. This protocol allows semi-quantitative assessment on sections [28].

The following rabbit primary antibodies were used: ASS (1:10,000) [15]; ARG1 and ARG2 (1:500, sc-20150 and 1:400, sc-20151, respectively, Santa Cruz Biotechnology, California); CPS (1:500) [29]; OAT (1:1,500) [30]. Antibody binding was visualized with alkaline phosphatase-coupled goat anti-rabbit IgG (1:200, Sigma A-3687). NOS1 and NOS3 were detected with mouse monoclonal antibodies (NOS1 1:400, IgG2a clone #16; NOS3 1:200, IgG1 clone #3, BD Transduction Laboratories) and visualized with an alkaline phosphatase-coupled goat anti-mouse secondary antibody (1:200, Sigma A3562) and a peroxidase-coupled rabbit anti-mouse secondary antibody (1:200, Sigma A3682), respectively. Single NOS1 positive cells in the lamina propria were further characterized by staining for the presence of CD68 (monocytes/macrophages 1:200, Dako M0876), CD3 (T-lymphocytes 1:600, Dako A0452), CD20 (B-cells 1:500, Dako M0755 clone L26), CD1A (dendritic cells 1:10, Neomarkers MS-1856-P1), and CD138 (plasma cells 1:100, Dako M7228 clone MI15).

Western Blotting

Villus epithelium was scraped off fresh duodenal resection material and lysed in SDS-PAGE sample buffer. After separation of samples on 10% SDS-polyacrylamide gels and blotting to PVDF membranes, proteins were visualized with antisera to ARG1 (1:200); ARG2 (1:500); NOS1 (1:2500); NOS3 (1:2500); α-SMA, (1:1000). The appropriate secondary horse-radish peroxidase-coupled antibodies were used at a dilution of 1:10,000. The signal was amplified using the chemiluminescent Super Signal West Pico reagent (Pierce, Perbio Science, The Netherlands) and pictures were taken with a LAS3000 imaging system (Fujifilm). α-smooth muscle actin was used to determine the contribution of the submucosa to the scrapings.

Evaluation of samples

Sections were scored for staining intensity in random sequence by 3 investigators with the readers of the slides blinded. Villi and crypts were scored separately. The intensity of ASS, OAT and ARG2 staining in enterocytes was expressed on a scale of 0–3 (0: absent; 1: weak; 2: intermediate; and 3: strong expression) relative to their expression in the ganglia of the myenteric plexus, which always contained both strongly and weakly positive neurons for ASS, OAT, and ARG2. Expression in the strongly staining neurons was set at 3. Samples without ganglia (mucosal biopsies from the duodenum) were compared to simultaneously stained samples that did contain ganglia to assign an intensity score from 0 to 3. Because CPS expression in the small intestine is restricted to enterocytes, all samples were stained simultaneously and then compared to each other using the same scale of 0–3 as described above. There was never more than one scoring unit difference between the 3 investigators.

Statistics

We used box-plots to visualize distribution and developmental changes of enzyme expression in enterocytes. Two-way analysis of variance (ANOVA) on rank-transformed data showed age-group and structure (crypt or villus) differences as well as age and structure interactions per enzyme. Sex (male or female) was not a factor associated with differences in enzyme expression. To pin-point which age-groups and crypts or villi differed, non-parametric one-way ANOVA (Kruskal-Wallis) tests were performed between age-groups per structure. Multiple comparison (Mann-Whitney) tests between structures per age-group were performed, when the null hypothesis was rejected. P values were considered significant if < 0.05.

Results

The earliest sample studied was a small intestine of a 14-week-old fetus, whereas the oldest sample was from a 79 year-old patient. Most of the samples examined were from patients younger than 7 years. At 14 weeks of gestation, all structural components of the epithelium, enteric nerves, and smooth muscle layers have formed [31,32]. Specimens were from the duodenum, jejunum, or ileum, with the majority of samples originating from the duodenum and ileum (Table 1). From 4 patients (age groups 5 and 6; 3–7 years), parallel samples of duodenum and ileum were available. In these samples, no differences in staining intensities of the enzymes investigated were observed between the proximal and distal small intestine or between males and females. For these 2 reasons, we felt justified to pool the samples of one age group. Of necessity, the studied specimens included both mucosal biopsies and full-thickness specimens, were all obtained to diagnose gastrointestinal conditions, and were contributed by different institutions. Because the gastrointestinal conditions were diverse in nature and the observed changes in enzyme levels concordant, our conclusions reflect developmental biology rather than pathology. Based on the staining patterns in these samples, we describe the developmental changes in the expression of arginine-synthesizing enzymes CPS, ASS and OAT, and the arginine-metabolizing enzymes ARG1, ARG2, NOS1 and NOS3 in the enterocytes of the small intestine.

Expression of CPS, ASS, OAT, ARG, NOS1 and NOS3 in enterocytes

Carbamoylphosphate synthetase (Figures 2A and 3; [see also Additional files 2 and 3])

CPS protein was exclusively found in the enterocytes. Prior to birth (group 1), CPS expression was uniformly

high in the enterocytes of both crypts and villi (Figure 3A). Between birth and 3 years of age (groups 2–4), CPS expression did not change significantly in the enterocytes on the villi, but declined thereafter to reach adult levels by 5 years of age (difference in villous expression between groups 1–4 vs. groups 5–8: $P \leq 0.0001$). Between birth and 1 year of age, expression was higher in the villus than in the crypt enterocytes ($P < 0.005$; Figure 3B [see Additional file 2]). Thereafter, staining differences between villi and crypts disappeared again (Figure 3C–F). No expression of CPS was found in the epithelium of Brunner's glands [see Additional file 3].

Ornithine aminotransferase (Figures 2B and 4; [see also Additional files 2 and 3])

OAT expression in the epithelium was uniform prior to birth. The youngest samples that could be investigated were from gestational week 23 (Figure 4A). Fetal expression was significantly higher in both crypts and villi than in the other age groups ($P \leq 0.004$). Directly after birth, OAT expression remained strong in the epithelium covering the villi, but declined in the crypts ($P \leq 0.017$), with the exception of age group 4 (1, 5-3 years). In some preparations, expression was stronger at the base of the villi, just above the crypts (Figure 4B, D). Above 40 years of age, OAT expression again resembled the fetal pattern, with crypt and villus enterocytes expressing OAT almost evenly albeit at a lower level (Figure 4E). The epithelium of Brunner's glands did not express OAT [see Additional file 3].

Argininosuccinate synthetase (Figures 2C and 5; [see also Additional files 2 and 3])

With the exception of the age groups 5 and 6 (3–7 years), ASS protein accumulated to a much higher concentration in the enterocytes on the villi than in the crypts ($P < 0.014$), especially before 3 years of age ($P < 0.004$). This pattern was already found in gestational week 14, where epithelial expression of ASS was still low (Figure 5A). From week 23 onward, expression in the villus epithelium was intermediate to strong. ASS expression remained high during the first postnatal year (groups 2 and 3 vs. groups 5–8, $P < 0.0001$), and then declined via an intermediate score between 1.5 and 3 years (group 4) to a near-absent score between 3 and 5 years (group 5). ASS expression in the crypts slowly declined after birth to become undetectable after 3 years of age (Figure 5B–F; [see also Additional file 2]) (Groups 1–4 vs. groups 5–8 in a multiple comparison of groups: $P < 0.0001$). As long as ASS expression was high (i.e. in the first 3 years), ASS protein was present throughout the enterocytes (Figure 5A–C), but in children and young adults, it gradually became concentrated at the basal side of the enterocytes (Figure 5D–F). No expression of ASS was found in the epithelium of Brunner's glands [see Additional file 3].

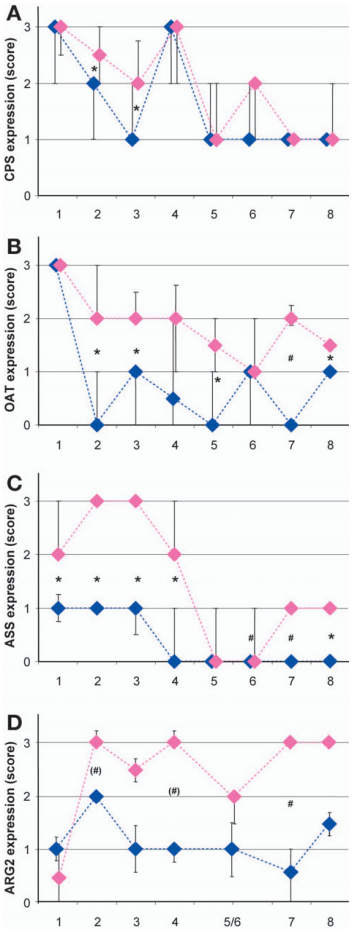

Figure 2
Developmental changes in expression of CPS, ASS, OAT and ARG2 in enterocytes of the small intestine. Sections represented in the top three panels (CPS, ASS and OAT) were stained simultaneously, whereas a subset of the samples with 2–4 individual sections per age group was stained later and is represented in the bottom panel (ARG2). The staining intensities were graded on a scale of 0 to 3. Medians of all observations of each age-group are indicated by diamonds and are plotted for villi (pink) and crypts (blue) separately. The vertical bars represent the first and third quartiles of each age group. When not drawn, the quartile coincides with the median. Age groups are: 1: fetus (14^{th}–39^{th} week of pregnancy); 2: 1–11 postnatal days; 3: 42–365 days; 4: 1.5–3 years; 5: 3–5 years; 6: 5–7 years; 7: 14–22 years; 8: 39–79 years; for details, consult Table 1. For CPS and ASS, expression in groups 1–4 was significantly higher than in groups 5–8, whereas for OAT, expression in prenatal group1 was higher than in the postnatal groups (for details, see main text). Significant differences in expression between villus and crypt enterocytes are indicated by an asterisk if $P \leq 0.005$, a "#" if $P \leq 0.017$, and a "+" if $P \leq 0.04$. Significant differences between age groups are described in the Results section.

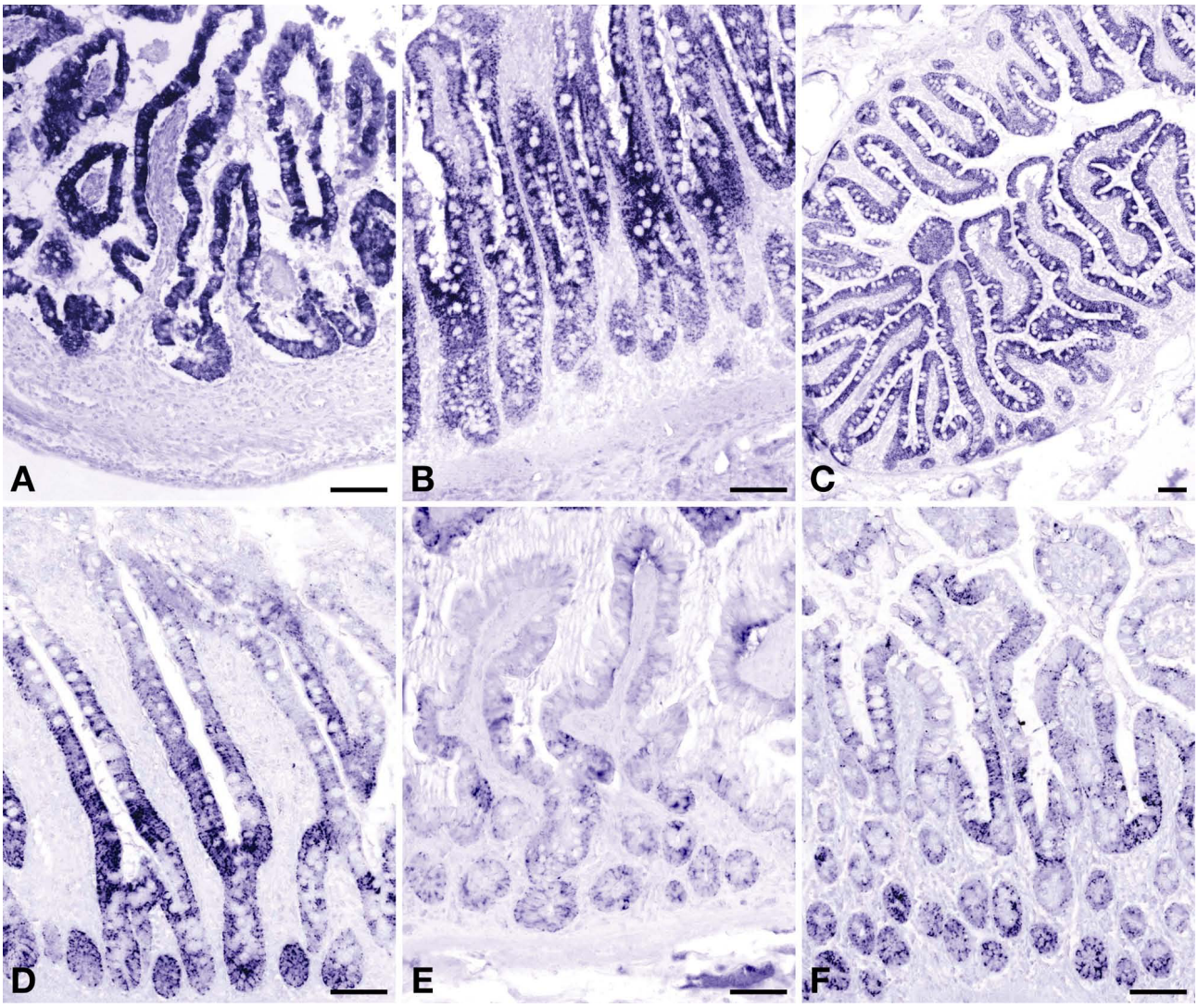

Figure 3
Developmental changes in expression of carbamoylphosphate synthetase in human small intestine. Panel A: gestational week 14; panel B: jejunum of a 3-day-old term male neonate; panel C: ileum of a 3-year-old male toddler; panel D: ileum of a 6-year-old male child; panel E: ileum of a 22-year-old female patient; panel F: ileum of a 70-year-old female patient. Scale bar: 100 μm.

Arginase-1 (Figure 6A)

ARG1, the major arginine-catabolizing enzyme, was not detectable immunohistochemically in the epithelium of the small intestine at any of the ages investigated. Western-blot analysis of samples from an adult duodenum (age: 60 years) were also negative for ARG1, confirming the immunohistochemical findings.

Arginase-2 (Figures 2D, 6A, and 7)

Western blots of the adult duodenum showed, instead, expression of the mitochondrial isoform of arginase (Figure 6A). We therefore investigated expression of ARG2 in a subset of samples (2–4/group) from all age-groups analyzed for ASS, CPS and OAT expression. ARG2 expression in fetal enterocytes was very weak (Figure 7A) or absent, but in neonates and all older age groups, expression was significantly higher ($P < 0.02$). After birth, protein expression in enterocytes was stronger on the villi than in the crypts (Figure 7B–F; $P < 0.0001$ for all groups in a multiple comparison; due to the relatively small sample size this difference was not significant for all individual groups).

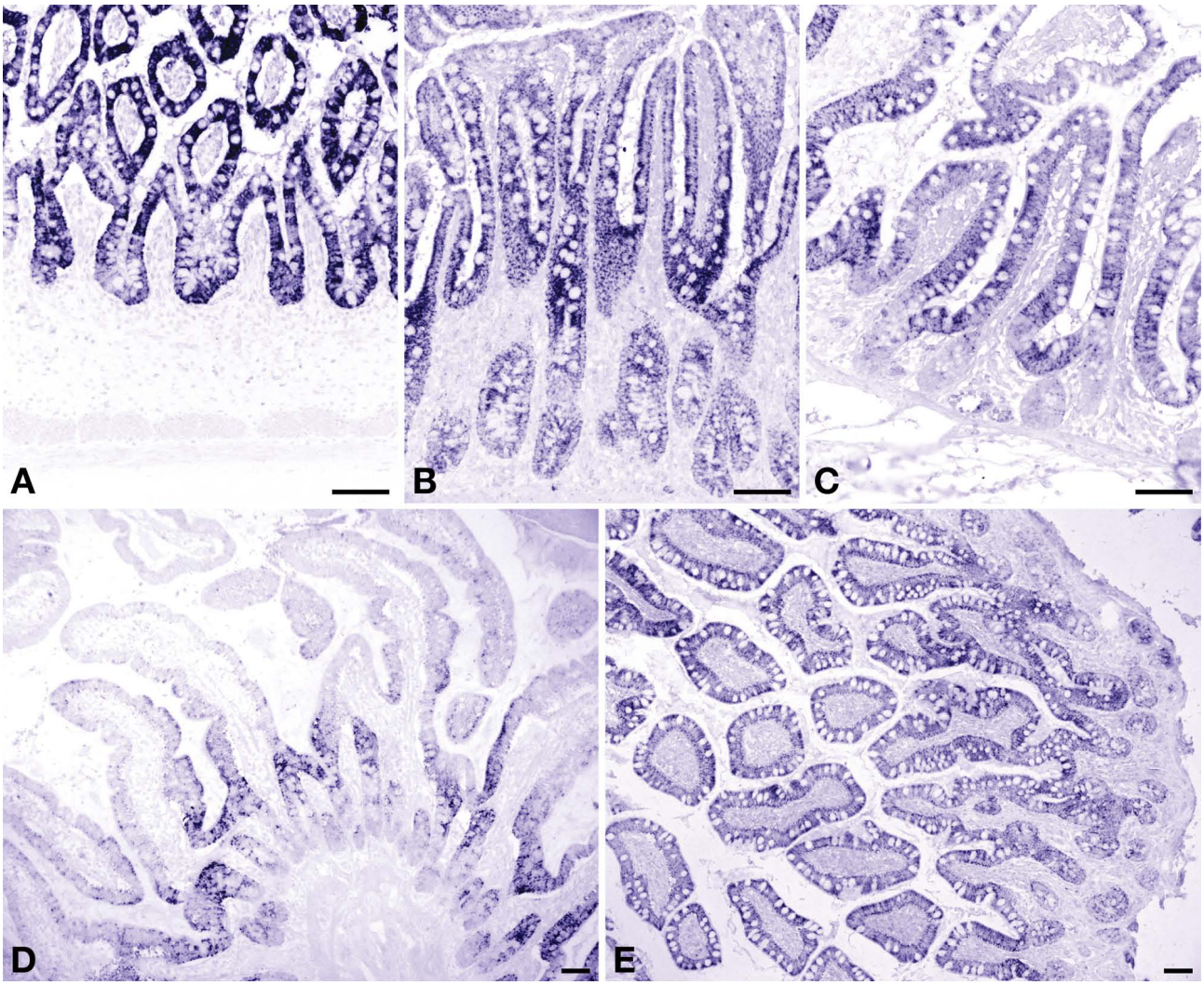

Figure 4
Developmental changes in expression of ornithine aminotransferase in human small intestine. Panel A: gestational week 23; panel B: jejunum of a 3-day-old term male neonate; panel C: ileum of a 3-year-old male toddler; panel D: ileum of a 6-year-old male child; panel E: ileum of a 70-year-old female patient. Scale bar: 100 μm.

NOS1 (Figure 6A)
Over-staining of sections with NOS1 yielded weak epithelial staining. Enterocytes of the human small intestine express high levels of NOS1 mRNA, but protein was not detected [33]. In agreement, a Western blot of a protein extract from villus epithelium did not show staining for NOS1, demonstrating that the observed staining of the sections was due to non-specific antibody binding.

NOS3 (Figure 6A)
We found weakly positive staining of villus epithelium with our NOS3 antibody. NOS3 mRNA is not expressed in enterocytes [33]. We, therefore, incubated a Western blot of a protein extract from epithelial cells with the same antibody as used for immunohistochemistry, but no immunoreactivity with NOS3 could be demonstrated, showing that the observed staining of the sections was due to non-specific antibody binding.

Localization of enzymes involved in arginine biosynthesis (Figure 8)
Serial sections of the distal duodenum at postnatal day 1 demonstrated that the highest expression of CPS, ASS, and OAT was found in the enterocytes covering the villi (Figure 8A, D, G). CPS-, OAT-, and ASS-positive enterocytes did not express ARG1 (not shown). The positive staining of the ARG1-rich lysed erythrocytes [34] inside vessels served as an internal positive control for the absence of ARG1 staining in enterocytes. ASS was also expressed in

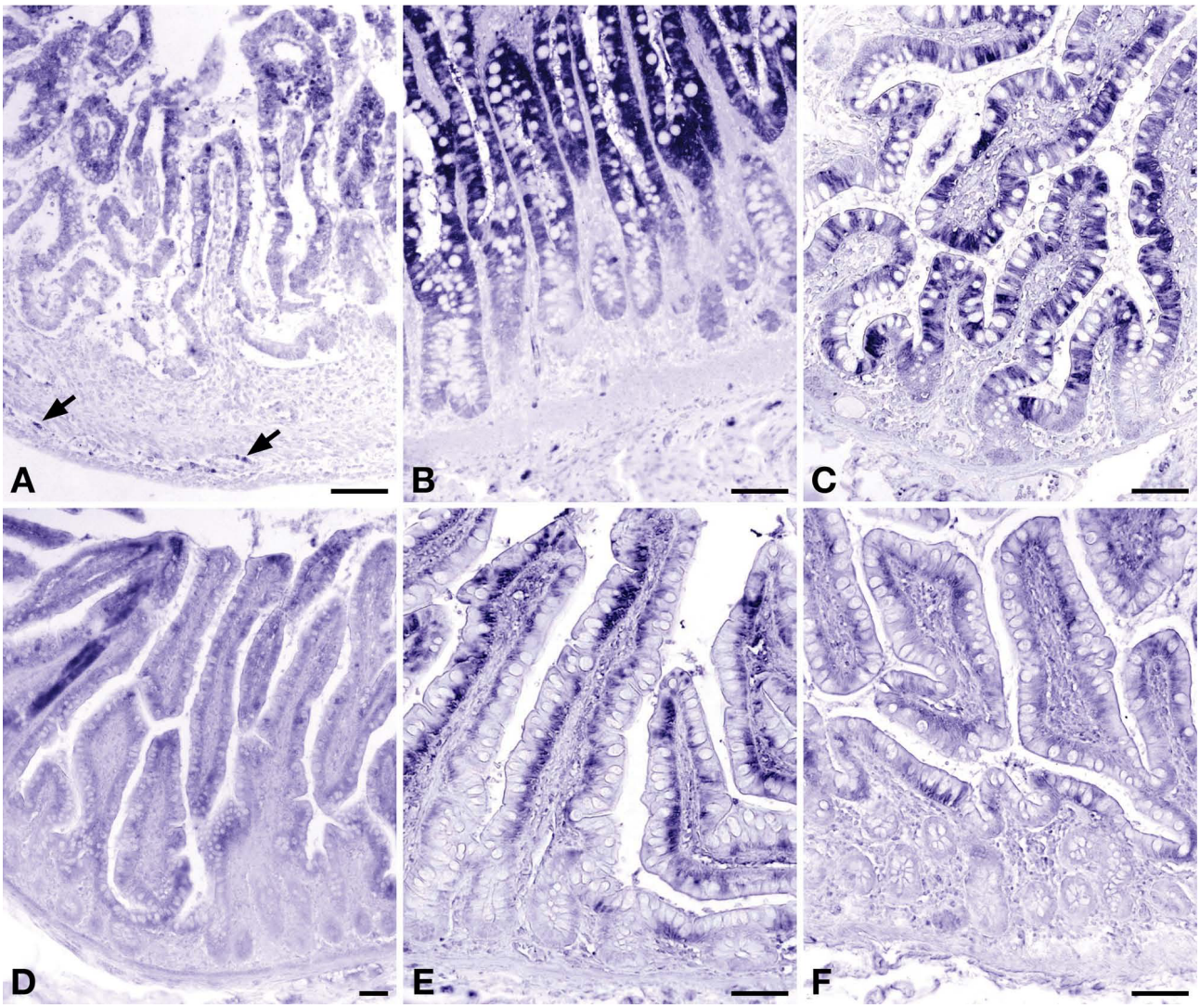

Figure 5
Developmental changes in expression of argininosuccinate synthetase in human small intestine. Panel A: gestational week 14; panel B: jejunum of a 3-day-old term male neonate; panel C: ileum of a 3-year-old male toddler; panel D: ileum of a 6-year-old male child; panel E: ileum of a 22-year-old female patient; panel F: ileum of a 70-year-old female patient. Arrows in panel A indicate ASS-positive neurons in the myenteric plexus. Scale bar: 100 μm.

the endothelium of the small arteries (Figure 8E, arrow), while OAT was present in the smooth muscle wall of these vessels (Figure 8H, white arrowhead). In addition, ASS and OAT were expressed in the ganglia of the myenteric plexus (Figure 8F, I, arrows). Only ASS was prominent in the varicose nerves of the circular muscle layer (Figure 8F, arrow head) and, to a lesser extent, in those of the longitudinal muscle layer.

Expression of enzymes in enteric nerves (Figure 6B)
Single neurons in the ganglia of both the myenteric and the submucosal plexus stained very strongly for ASS, OAT, ARG2, and NOS1, whereas other neurons in the same ganglion stained weaker or not at all. The strongly OAT-positive neurons were also positive for ASS and NOS1 (Figure 6B, black arrows), but some ganglia that were positive for both NOS1 and ASS did not express OAT (Figure 6B, white arrows). ASS- and NOS1-negative neuronal bodies were not observed in ganglia. Considerable staining of varicose nerve fibers for ASS and NOS1, but not for OAT, was observed in the circular and, to a lesser extent, the longitudinal smooth-muscle layer (Figures 6B and 8F). Positive staining for ASS of single neurons in myenteric ganglia was already found in gestational week 14

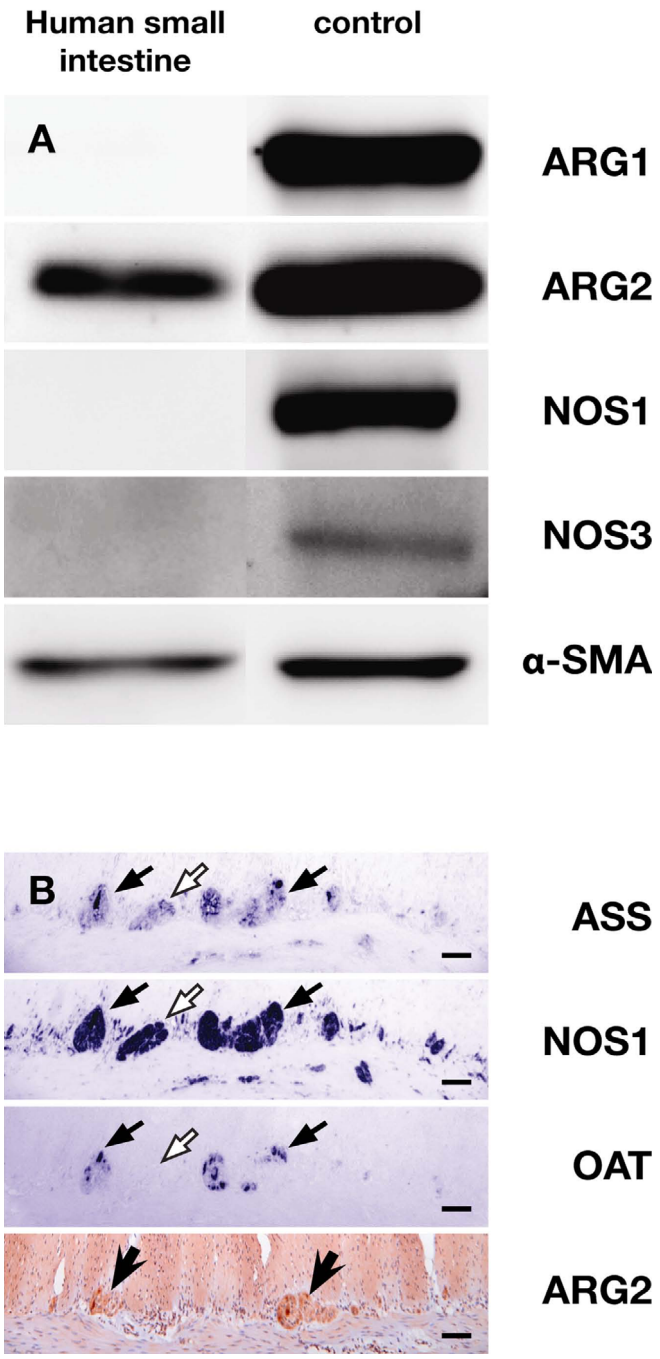

Figure 6
Expression of ASS, ARG and NOS in villus epithelium and neurons. Panel A: 40 μg of protein isolated from small-intestinal scrapings of a 60-year-old patient were loaded per lane (left column). Positive controls (right column) included 25 μg of human liver extract for ARG1, NOS1 and α-SMA, 25 μg of human kidney extract for ARG2, and 25 μg of mouse brain extract for NOS3. Absence of ARG1, NOS1 and NOS3 expression in the adult small-intestinal enterocytes is demonstrated. The density of the ARG2 band is approx. 10% of that in kidney. Panel B: Serial sections (5 μm) of the ileal myenteric plexus of a 6-year-old male child were stained for ASS, NOS1, and OAT. Note colocalization of ASS, NOS1, and OAT in intensely staining neurons (black arrows). Also note OAT-negative ganglia (white arrows). Another section of the same specimen was stained for ARG2. Scale bar: 100 μm.

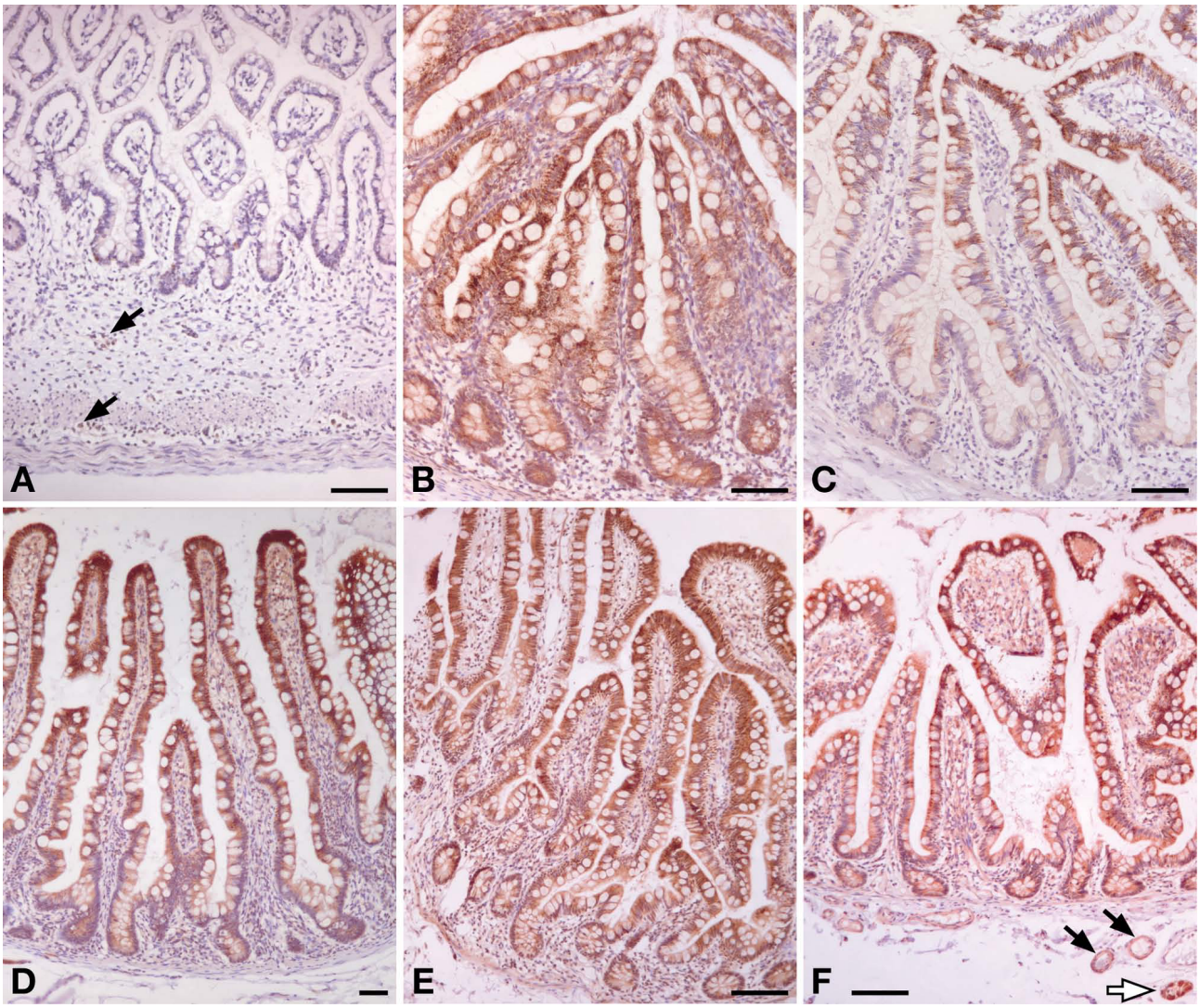

Figure 7
Developmental changes in expression of arginase-2 in human small intestine. Panel A: gestational week 23; arrows indicate positive neurons in submucosal and myenteric plexus; panel B: jejunum of a 3-day-old term male neonate; panel C: ileum of a 3-year-old male toddler; panel D: ileum of a 6-year-old male child; panel E: ileum of a 22-year-old female patient; panel F: ileum of a 70-year-old female patient; the white arrow shows staining of a submucosal ganglion, the black arrows indicate staining of smooth muscle cells in the wall of arterioles. Scale bar: 100 μm.

(Figure 5A, arrows), whereas NOS1 (data not shown) and ARG2 were detectable in gestational week 23 (Figure 7A, arrows). ASS, OAT, and ARG2 expression was also found in ganglia of the outer and inner submucosal plexus (Figures 6B and 7F). The neurons of the myenteric plexus stained stronger than those of the submucosal plexus, except those positive for ARG2, which stained equally strong in both.

Expression of enzymes in the wall of intestinal vessels

The endothelium of the small arterioles in the submucosa and serosa always stained positive for the presence of ASS (Figure 8E, arrow) and NOS3, but that of the larger arteries, veins and lymph vessels was negative for both enzymes. After birth, OAT and ARG2 expression was demonstrable in the smooth-muscle cell layer of arterioles (Figures 7F). No expression was found in the smooth-muscle cell layer of veins.

Expression of enzymes elsewhere in the intestine

Single cells in the lamina propria that were strongly positive for NOS1 were also positive for CD68, a marker for macrophages, but not for CD3, CD20, CD138, or CD1A (lymphocytes and dendritic cells (not shown). Germinal

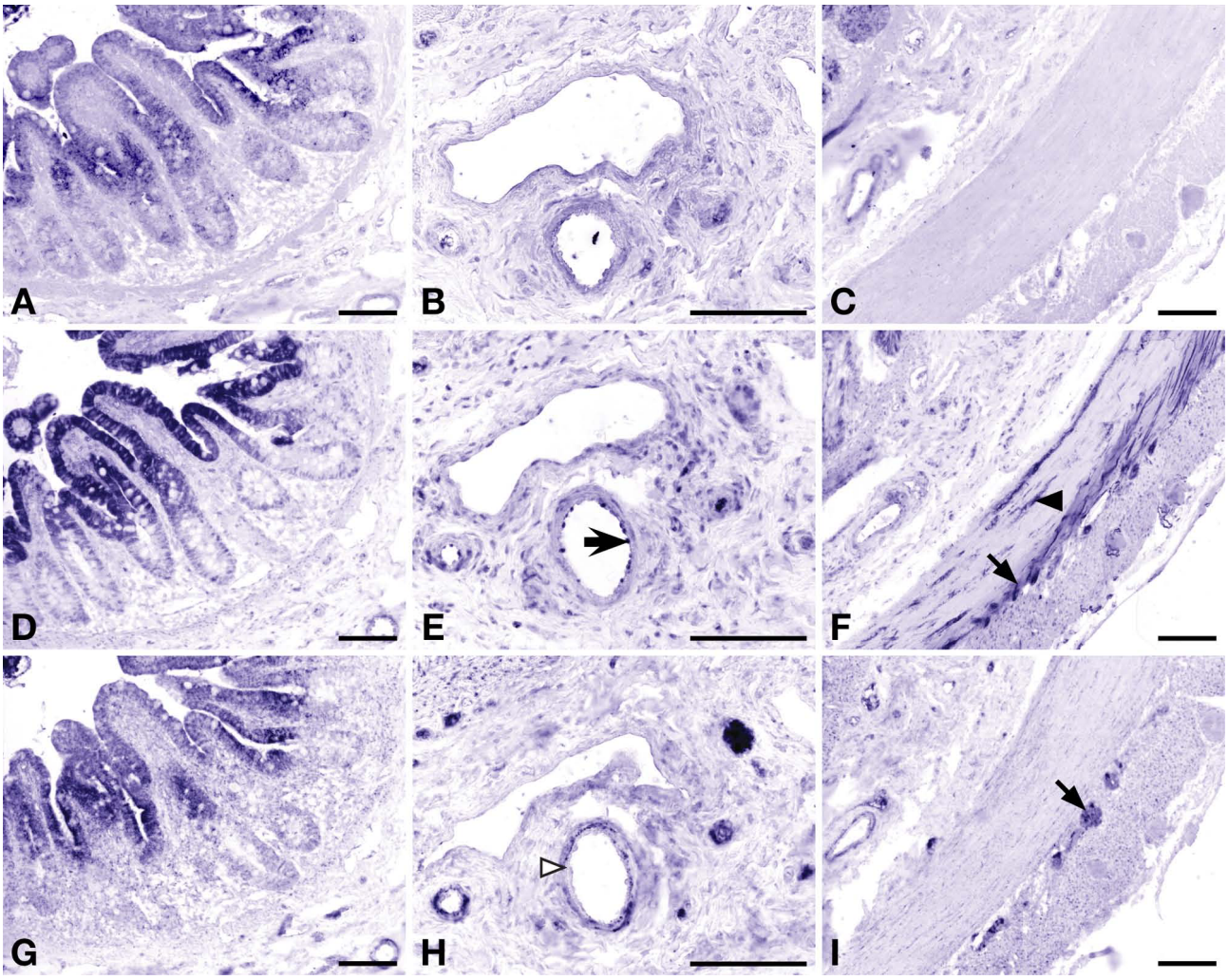

Figure 8
Expression of enzymes involved in arginine synthesis in human small intestine at postnatal day 1. Serial sections of the distal duodenum of a 1-day-old female neonate. Panels A-C show the expression of CPS; panels D-F: ASS; and panels G-I: OAT. Endothelial expression of ASS in panel E is indicated by block arrows, while OAT expression in the smooth muscle layer of the same vessels is indicated by a white arrowhead (panel H). Panels C, F and I show the muscularis propria with the inner circular and outer longitudinal layer. Myenteric ganglia are indicated by black arrows in panels F and I. Varicose nerves in the circular muscle layer are indicated by an arrowhead in panel F. Scale bar: 100 μm.

centers within lymphocyte aggregates also stained positive for NOS1 (not shown).

Discussion

The main result of this study is the observation that the epithelium of the perinatal human small intestine expresses the enzymes that exert control over the biosynthesis of arginine from proline, bicarbonate, and ammonia, viz. CPS, OAT and ASS (See Figure 1), and that the major arginine-degrading enzyme ARG1 is absent during that period. We deduced this conclusion from an enzyme-histochemical analysis of 79 specimens less than 8 years old.

The human small intestine expresses key enzymes of arginine synthesis at midgestation

Although the developmental appearance of CPS, OAT, and ASS in the enterocytes of the piglet and rodent small intestine has been reported [6-9,35-38], such information was only incompletely available for the human small intestine. CPS expression in the human small intestine starts as early as the 8th week of gestation [23,24]. Accordingly, CPS was expressed in the enterocytes of all our samples. To our knowledge, OAT expression in human enterocytes has not yet been reported, but its developmental profile resembles that of CPS, ornithine car-

bamoyltransferase and pyrroline-5-carboxylate reductase in fetal piglets [37]. In agreement, OAT was, like CPS, expressed in the enterocytes of all our samples. ASS, finally, is expressed in Caco2 cells [39], but its expression in normal human small intestinal enterocytes has not yet been reported. ASS differed from CPS and OAT in that its expression declined profoundly between 3 and 5 years of age (Figure 2). The time course in ASS expression in the postnatal human gut resembles that in piglets, which declines towards weaning and then rises again [10]. In rodents, on the other hand, ASS expression disappears completely at weaning [6,40]. Concurrent with the developmental decline in ASS expression, its homogeneous cytosolic distribution changed to one that is restricted to the basal side of the enterocytes (Figure 5E, F). Asymmetric localization of proteins in enterocytes has been previously described as a consequence of mRNA sorting [41], but a change in enzyme localization during late postnatal development has, to our knowledge, not yet been demonstrated.

The crypt-villus gradient of postnatal CPS and ASS expression in the suckling human intestine resembles that in piglets (our unpublished observations), but markedly differs from that in rodents. In both rats and mice [5,6], ASS expression is confined to the enterocytes occupying the apical half of the villi, whereas CPS is expressed in all enterocytes. This expression pattern suggested to us that the basal enterocytes synthesized citrulline, whereas the apical enterocytes synthesized arginine [6]. A possible explanation for this spatial separation of cells capable to produce citrulline only and cells also capable of arginine production is that the small-intestinal enterocytes are the only cells in the body that can synthesize citrulline and that this citrulline is necessary as substrate for arginine synthesis elsewhere, e.g. the kidney and the endothelium. Apparently, such a zonation of arginine metabolism is not necessary in newborn pigs and humans.

The net production of ornithine by the small intestine is a prerequisite for citrulline and arginine biosynthesis [42]. The severe deficiency of circulating ornithine and arginine that develops in neonates lacking OAT [38,43] demonstrates that, in the neonatal intestine, OAT indeed serves to produce ornithine. Citrulline is exported from the mitochondria in exchange for ornithine via the ornithine carrier ORNT. Accordingly, the ORNT1 isoform is expressed at elevated levels in the small intestine of the mouse during the suckling period [44]. However, the relatively high expression of ARG2, the mitochondrial isoform of arginase, in the neonatal and adult human small intestine could potentially nullify the cytosolic synthesis of arginine (in the mouse small intestine, ARG2 becomes only expressed at weaning [5,6]). Extensive degradation of cytosolic arginine due to import into the mitochondria is, nevertheless, unlikely, because the affinity of arginine for the ORNT carrier is ~10-fold lower than that of ornithine [45].

Together, our findings indicate that the human fetal intestine has acquired the potential to produce arginine at 23 weeks of gestation and probably as early as 14 weeks, that is, well before fetuses become viable outside the uterus. Furthermore, NOS1, NOS3 and their downstream target soluble guanylate cyclase (not shown) were expressed at term levels in fetuses of ~23 weeks of pregnancy. This indicates that at this time in gestation the fetus not only has the potential to synthesize arginine, but also to use it for NO and cyclic GMP production. Hypoargininemia, nevertheless, often develops in preterm infants, in particular if they are maintained on total parenteral nutrition [5,6,15], and appears to predispose them to the respiratory distress syndrome [46] and necrotizing enterocolitis [19,20]. The hyperammonemia that frequently accompanies hypoargininemia in preterms responds to intravenous arginine supplementation [18], which indicates that endogenous arginine biosynthesis is deficient. The strong association with parenteral nutrition points to the intestines and indicates that the intestines only produce arginine if substrate is supplied from the intestinal lumen, as was shown for the newborn piglet [11,12].

Intestinal neurons and arterioles express key enzymes of arginine synthesis

The neurons of the myenteric plexus abundantly express OAT, ASS, and NOS1. Since the neurons do not express CPS and, therefore, cannot synthesize citrulline from ornithine, OAT most likely functions to produce glutamate, while ASS probably functions to (re-)synthesize arginine from citrulline [47]. It is conceivable that these neurons require under certain conditions additional arginine (synthesized by enterocytes) above their endogenous production. Such metabolic cooperation has been demonstrated for the intestinal sphincters: neurons in these sphincters can resynthesize arginine from citrulline, but become dependent on external arginine during prolonged activity [48]. The expression pattern of soluble guanylate cyclase (not shown) demonstrated that there are many target cells for the NO produced by the neurons of the myenteric plexus. In this respect, the inner, circular layer of the muscularis propria differed markedly from the outer, longitudinal layer both by the far more abundant distribution of ASS- and NOS1-positive nerve fibres and its much stronger expression of soluble guanylate cyclase (not shown). The much stronger immunoreactivity for NOS1 of the nerve fibers in the circular muscle has been described [49]. Although nerve cell content and density of the NOS-positive ganglia of the myenteric plexus [49-51] markedly declines in the peri- and postnatal period, this structural difference between both layers of the muscularis propria is maintained throughout development.

The constantly fed state of the neonatal small intestine causes absorptive hyperemia, that is, a high intestinal blood flow and a low intestinal vessel resistance [52]. NO production in the vessel wall is the main determinant of this vascular relaxation [53], in particular during the suckling period [54]. Since neonatal hypoargininemia is closely associated with the risk to develop necrotizing enterocolitis, a disease of the intestinal vessels [55], we speculate that arginine biosynthesis in both the enterocytes and the endothelial cells of the small vessels of the intestinal submucosa is necessary to support this hyperemic state and to protect the neonatal intestine from ischemia. Unfortunately, only one intervention trial [56] without a conclusive outcome [57] has tested the predicted beneficial effect of arginine supplementation to preterm neonates thus far. Although arteries of the 1st to 2nd order (out of 4 size categories) are considered to be the major sites of resistance and blood flow regulation of the gut [58], we did not observe ASS and NOS3 expression in the endothelium of 1st order (largest) arteries. Furthermore, the downstream target of NO, soluble guanylate cyclase, was found in the smooth-muscle layer of arterioles only (not shown), suggesting that these 2nd order vessels materially contribute to the peripheral vascular resistance in the gut. A recent study showed a deficiency in NO production but not in NOS3 expression as judged by immunohistochemistry of intestinal arterioles of patients with NEC [59], supporting our hypothesis that NOS substrate deficiency plays a role in intestinal ischemia. In addition to NO production, other factors are involved in neonatal vascular dysfunction, such as the pro-inflammatory cytokine IL-1β and endothelin-1 [60], but their effects are mediated at least in part via a blunting of NOS3-mediated NO production.

The decline of arginine-synthesizing enzymes in enterocytes and "weaning"

The age-dependent postnatal decline in the expression of ASS in the enterocytes of the human small intestine is most pronounced between 3 and 5 years of age. The time course in ASS expression in the developing human gut resembles that in piglets, where activity is highest during the suckling period, declines to low levels around weaning (under natural conditions occurring at 12–15 weeks [61]) and then rises again [10]. In rodents, on the other hand, ASS expression disappears completely at weaning [5,6,15]. In humans, approximately 6 months of age is considered to be an optimal time point for weaning, but the natural weaning age for humans may be as late as 2.5–3 years [25]. The prominent reduction of ASS expression that we observed after three years of age, therefore, coincides with this assumed natural weaning age.

There is a remarkable similarity in the developmental timing of the decline in expression of ASS and lactase-phlorizin hydrolase (hereafter called lactase), another small-intestinal enzyme that is closely associated with breast-feeding. In rodents, lactase expression declines to undetectable levels at weaning [62], whereas in piglets, the decline occurs more gradually during the first 8–16 weeks of life [63]. In lactase-nonpersistent humans, lactase activity begins to decline between 2 and 3 years [64]. The temporal coincidence of the intestinal capacity to digest lactose and to produce arginine does support a relation to milk as the main source of food and underscores the notion that mammalian milk does not contain enough arginine to support rapid postnatal growth [17], so that intestinal arginine synthesis is necessary. Although we did find low levels of ASS in adult intestine, it was recently reported that the human intestine (age range 37–69 years) does not produce arginine [65]. This finding does not exclude a role for local arginine synthesis in e.g. neurons and endothelial cells that is directly coupled to NO production within the same cell.

Conclusion

Our data show that CPS, ASS, and OAT expression is strong in the enterocytes of fetuses, neonates, infants and toddlers. Humans, therefore, resemble other mammals in that the enterocytes of their small intestine are major producers of arginine during the suckling period. Although the relative deficiency of arginine in milk seems to underlie this temporary function of the gut, the intestinal or systemic functions that require the arginine that is produced in the gut remain to be delineated. We submit that relaxation of the circular smooth muscle layer of the muscularis propria [49,66] and that of the intestinal arterioles [54] stand out in this respect.

Abbreviations

ARG: arginase (E.C. 3.5.3.1); ASS: argininosuccinate synthetase (E.C. 6.3.4.5); CPS: carbamoylphosphate synthetase (E.C. 6.3.4.16); NOS: nitric oxide synthase (E.C. 1.14.13.39); OAT: ornithine aminotransferase (E.C. 2.6.1.13).

Authors' contributions

EK participated in the design of the study and carried out the data analysis and drafted the manuscript. SS assisted in data analysis and processing. CG assisted in the study design, data analysis and critically revised the manuscript. PD carried out immunoassays. JV carried out immunoassays. JR assisted in the design of data analysis and performed the statistical analysis. WL conceived of the study, participated in analysis and interpretation, and critically revised the manuscript for important intellectual content. EB is the pediatric pathologist of this study, was responsible for the diagnoses and data interpretation and critically revised the manuscript.

Additional material

Additional file 1

Representative negative controls.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-213X-8-107-S1.pdf]

Additional file 2

Expression of ASS, CPS and OAT in a group 3 patient.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-213X-8-107-S3.pdf]

Additional file 3

Brunner glands in a group 5 patient.
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[http://www.biomedcentral.com/content/supplementary/1471-213X-8-107-S2.pdf]

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CHAPTER V

HEPATIC ADAPTATION COMPENSATES INACTIVATION OF INTESTINAL ARGININE BIOSYNTHESIS IN SUCKLING MICE

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Hepatic Adaptation Compensates Inactivation of Intestinal Arginine Biosynthesis in Suckling Mice

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Abstract

Suckling mammals, including mice, differ from adults in the abundant expression of enzymes that synthesize arginine from citrulline in their enterocytes. To investigate the importance of the small-intestinal arginine synthesis for whole-body arginine production in suckling mice, we floxed exon 13 of the *argininosuccinate synthetase* (*Ass*) gene, which codes for a key enzyme in arginine biosynthesis, and specifically and completely ablated *Ass* in enterocytes by crossing Ass^{fl} and *Villin-Cre* mice. Unexpectedly, $Ass^{fl/fl}/VilCre^{tg/-}$ mice showed no developmental impairments. Amino-acid fluxes across the intestine, liver, and kidneys were calculated after determining the blood flow in the portal vein, and hepatic and renal arteries (86%, 14%, and 33%, respectively, of the transhepatic blood flow in 14-day-old mice). Relative to control mice, citrulline production in the splanchnic region of $Ass^{fl/fl}/VilCre^{tg/-}$ mice doubled, while arginine production was abolished. Furthermore, the net production of arginine and most other amino acids in the liver of suckling control mice declined to naught or even changed to consumption in $Ass^{fl/fl}/VilCre^{tg/-}$ mice, and had, thus, become remarkably similar to that of post-weaning wild-type mice, which no longer express arginine-biosynthesizing enzymes in their small intestine. The adaptive changes in liver function were accompanied by an increased expression of genes involved in arginine metabolism (*Asl*, *Got1*, *Gpt2*, *Glud1*, *Arg1*, and *Arg2*) and transport (*Slc25a13*, *Slc25a15*, and *Slc3a2*), whereas no such changes were found in the intestine. Our findings suggest that the genetic premature deletion of arginine synthesis in enterocytes causes a premature induction of the post-weaning pattern of amino-acid metabolism in the liver.

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Introduction

Arginine is a substrate for the synthesis of nitric oxide, urea, ornithine, citrulline, creatine, agmatine, glutamate and proline [1]. The rate-determining enzyme of arginine synthesis from citrulline and aspartate is argininosuccinate synthetase (ASS) (EC 6.3.4.5) [2]. The endogenous biosynthetic capacity for arginine suffices to meet the daily requirement under normal conditions, but a dietary source of arginine becomes necessary when demand increases under anabolic or catabolic conditions [3–5]. For this reason, arginine is a conditionally essential amino acid.

These general conclusions apply to post-weaning individuals that can adapt their sources of food. However, arginine metabolism in the early postnatal period differs considerably from that in the adult, probably because the rapidly growing suckling animals have no choice of food. Since the supply of arginine via the milk is not sufficient to support optimal protein synthesis [6–8], suckling animals need a higher endogenous capacity for arginine synthesis. The abundant expression of the enzymes necessary for *de novo* arginine biosynthesis in the enterocytes of the small intestine of perinatal rodents [9–11], piglets [12,13] and humans [14] and the absence of cytosolic arginase expression [9,15–18] suggest that the

intestine is an important site for early postnatal arginine production in mammals. In fact, it has been demonstrated that the small-intestinal mucosa accounts for ~50% of the endogenous production of arginine in normally fed suckling piglets, while hypoargininemia develops if this source of arginine production is bypassed as during parenteral feeding [19–21]. In addition, we have shown that suckling mice that transgenically overexpress *arginase1* in the small-intestinal enterocytes suffer from deficient hair and muscle growth, and underdevelopment of Peyer's patches due to impaired B-cell maturation [15,16]. Interestingly, the capacity of the intestine to produce arginine becomes limited to citrulline synthesis in the weaning period, when the expression of argininosuccinate synthetase and lyase in the intestines declines in humans and pigs [14,17,18], and stops in rodents [9]. Although these findings suggest that intestinal arginine biosynthesis is essential for suckling animals, this hypothesis remains to be tested.

To assess to what extent the small-intestinal arginine synthesis is essential for suckling mice, we have eliminated the expression of argininosuccinate synthetase (*Ass*) in the enterocytes, using the Cre-loxP recombinase system. Since the total-body deletion of *Ass* is lethal [22], exon 13 of the *Ass* gene was surrounded by loxP sites. Enterocyte-specific excision of the "floxed" exon was accomplished by breeding Ass^{fl} mice with *VilCre* mice [23]. Suckling $Ass^{fl/fl}/VilCre^{tg/-}$ mice (*Ass*-KO/I) were completely devoid of *Ass* expression in their enterocytes, but unexpectedly did not suffer from growth retardation or any other detectable functional impairment. Whereas *Ass* elimination from enterocytes did not cause adaptive changes in gene expression in enterocytes, it did change the expression of several amino acid-metabolizing and transporting genes in the liver with as result that arginine deficiency did not develop. Our findings highlight a homeostatic regulatory interaction of the gut-liver axis with respect to arginine metabolism.

Methods

Plasmid construction and recombinant ES cell selection

The mouse *Ass* gene is located on chromosome 2. The targeting vector (14.384 Kb) consisted of the 3' 4.2 Kb of intron 12, a Neo-TK selection cassette flanked by *frt* sites, exon 13 flanked by *loxP* sites and the 5' 3.5 Kb of intron 13 (Figure S1A). The targeting construct was sequence verified with respect to splice junctions, exon 13 and recombinase-recognition sites, digested with AscI and PmeI (introduced by PCR for cloning purposes), and purified by electrophoresis and electro-elution. Twenty-five µg of the targeting fragment was electroporated into the 129P2/OlaHsd-derived mouse ES cell line E14IB10, a subclone of E14 cells [24]. Selection with G418 (200 µg/mL) was started 24 hours after electroporation. Short vector sequences that were left on either end of the targeting construct allowed a PCR-based negative screen against random integrations (Figure S1B, upper panels). Long-distance PCR with an *Ass*-specific primer outside the targeting construct and a *loxP*-specific primer was used to demonstrate homologous recombination at the 3' side in the remaining clones (Figure S1B, middle panel). Proper recombination of the 5' end was demonstrated with Southern blotting and probing with a Neo fragment (Figure S1B, lower panel). The Neo-TK cassette was removed by transient transfection with an *FLPe* recombinase expression vector (kindly provided by Dr. Francis Stewart, EMBL, Germany). Selection with ganciclovir (5µM) was started 5 days after electroporation. The recombined allele was again sequence verified (ganciclovir introduced a mutation in 1 of 4 clones tested). Two clones containing 40 chromosomes were selected for blastocyst injection.

Generation of transgenic mice and husbandry

Ass-recombinant ES cells were injected into C57BL/6J blastocysts. Chimeric male mice were bred with female 129P2/OlaHsd mice (Harlan, The Netherlands) and with deleter-Cre females on a C57BL/6 background (kindly provided by Dr. Ursula Lichtenberg, University of Cologne, Germany) to delete *Ass* exon 13 in the germ line [25]. Mice were genotyped with primers *Ass-F1* and *Ass-R1* (Table S1 Figure S1C and D, upper subpanel), yielding a 360 bp wild-type allele and a 390 bp floxed allele. The Cre-excised allele (340 bp) was detected by PCR with the primers *Ass-F2* and *Ass-R1* (Figure S1C and D, middle subpanel). To specifically delete the Ass^{fl} allele in the enterocytes of the small intestine, mice were crossed with *VilCre* mice [23]. Animals were genotyped for the presence of *VilCre* by PCR with primers *Vil-F* and *Vil-R* (see: Table S1 and Figure S1D, lower subpanel), yielding a 1,100 bp band for *VilCre*-positive animals. All mice used in this study except the controls in Figure 1A had the same genetic background.

Animals were housed in humidity- and temperature-controlled rooms on a 12-hour light/ 12-hour dark cycle with food and water ad libitum. The animal studies were reviewed and approved by the institutional Animal Care and Ethical committee (DEC) of Maastricht University.

Blood and tissue collection

Animals were sacrificed by decapitation on neonatal days (ND) 14, 18, 21, and 35. Blood samples from the hepatic vein, portal vein, renal vein and left ventricular cavity were taken from 14- and 35-day-old mice under 2% isoflurane (Abbott, # B506)/ 90% O_2 anesthesia, as described for adult mice [26]. Tissues were fixed in 4% buffered formalin and embedded in Paraplast®.

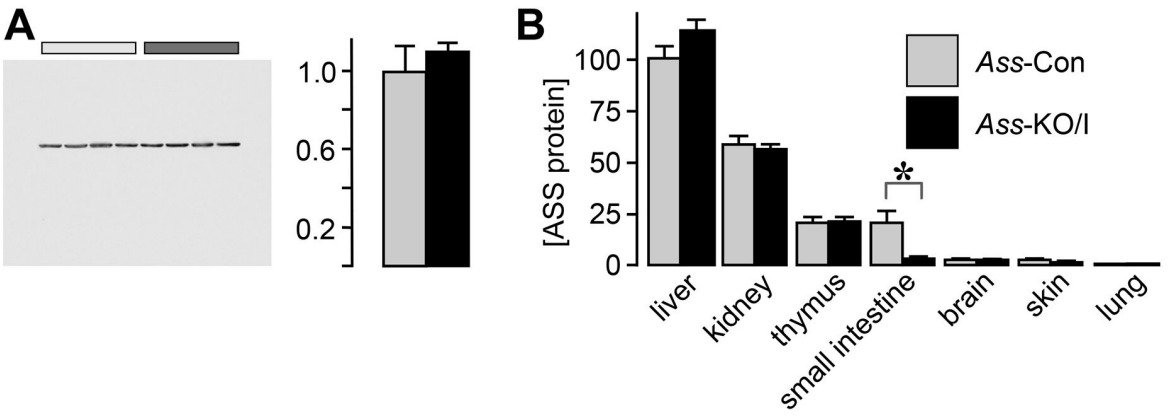

Figure 1. Expression of the *Ass*^{fl} allele is not compromised and allows efficient excision. Panel A: Western blot demonstrating that the *loxP* sites surrounding exon 13 do not affect ASS expression at the protein level (liver extract with 25µg total protein was loaded per lane; quantification of band intensity on right subpanel). Panel B: ASS protein in ND14 *Ass*-KO/I mice is eliminated in the enterocytes of the small intestine only. Note that the residual ASS in the small intestine reflects the presence of the ASS-positive enteric ganglia (Figure 2). Light gray bars indicate *Ass*-Con mice and dark gray bars *Ass*-KO/I mice (N=4 each).
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Amino-acid concentration

Blood was collected into heparin-coated tubes and centrifuged at 2,000*g for 5 min at 4˚C. 80 µL of plasma was added to 6.4 mg of lyophilized sulphosalicylic-acid, vortexed and stored at -20˚C. Amino-acid concentrations were determined by HPLC as described [27]. Amino-acid fluxes were calculated as described [26]. Because only the relative distribution of flows across organs could be measured (next section), fluxes were expressed as the product of the arterio-venous differences in amino-acid concentrations and the relative flow across the respective organs.

Blood flow distribution using radioactive microspheres

Radioactive microspheres (15 µm diameter), labeled with ^{103}Ru, were purchased from PerkinElmer USA (NEM082A100UC). Fourteen-day-old mice (6-8 gram body weight) were anesthetized by intraperitoneal injection of 100 µg/g ketamine (Pfizer) + 20 µg/g xylazine. After opening the thorax through the fourth left intercostal space, 50 µL of a 0.01% Tween-80/ saline solution containing 10,000 microspheres (~20 KBq) was injected into the left ventricle. Organs were isolated, transferred to a plastic counting vial containing 2 mL of saline and measured with a gamma counter (Wallac Wizard 1480; PerkinElmer, USA).

Western blotting and histology

Tissues were crushed in a liquid nitrogen-cooled mortar, sonicated in RIPA buffer (25 mM Tris·HCl(pH=7.6), 150 mM NaCl, 1% NP-40, 1% Na-deoxycholate, 0.1% Na-dodecylsulfate), containing a protease inhibitor cocktail (Complete; Roche, The Netherlands). Twenty-five µg of protein was loaded per lane. Equal protein loading per lane was verified by Ponceau-S staining of the blots prior to incubation with antiserum. ASS was visualized by incubating the blots with rabbit anti-mouse ASS antiserum (1:1,500 [15,16]), followed by alkaline phosphatase-coupled goat anti-rabbit antibody and "Super Signal West Femto Maximum Sensitivity Substrate" (Pierce # 34095).

Duodenal (2 cm distal to the pyloric sphincter) and ileal (2 cm proximal to the ileocecal junction) segments were fixed in ice-cold buffered 4% formaldehyde, embedded in Paraplast®, and sectioned at 7 µm. Goblet cells were visualized with 1% Alcian blue (BDH, The Netherlands) in 3% acetic acid (pH = 2.5) for 30 min. Nuclei were visualized with 1% Nuclear Fast Red (Chroma, Fluka) for 3 min. For immunostaining, sections were subjected to antigen retrieval (10 min at 100°C in 10 mM Na-citrate (pH 6.0)), blocked with Teng-T (10 mM Tris (pH=7.6), 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20) /10% normal goat serum (NGS, Gibco, The Netherlands) for 30 min, and incubated overnight in a 1:1,000 dilution of the ASS antibody [15,16] in Teng-T/10% NGS. After washing with PBS and another blocking step with Teng-T /10% NGS for 15 min, sections were incubated with goat anti-rabbit antiserum (Sigma A3687; diluted 1:200 in Teng-T /10% NGS) for 90 min. After washing, the slides were incubated with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/ BCIP; Roche 169471) at 37˚C until staining reached an optical density not exceeding 0.8.

Visualization of Peyer's patches

The small intestine was immersed for 5 min in 10% acetic acid (v/v) before fixation in 10% neutral-buffered formalin, as described previously [16].

PCR assays

RNA was extracted from cells and tissues with guanidinium thiocyanate (TRI Reagent, Sigma). RNA integrity was assessed by denaturing gel electrophoresis and RNA concentration with a NanoDrop-ND1000 spectrophotometer (Isogen Life Sciences). One µg of total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). The cDNA preparation was diluted 1:10 (v/v) with milliQ water prior to addition to the PCR mixtures.

Quantitative PCR. PCR was performed using iQTM SYBR® Green Supermix (Biorad 170-8882) in an iQ5 real-time PCR detection system (Biorad). The extension temperature was 60˚C for all primer pairs. cDNA samples were diluted 60-fold before use for mRNAs and 600-fold for 18S rRNA. Primary fluorescent data were exported and analyzed using the Lin-RegPCR program [28]. All primers (Table S1) were obtained from Genosys (Sigma).

PCR array. TaqMan® OpenArray® Real-Time PCR Plates containing validated primers for mRNAs of 42 different genes involved in arginine metabolism or transport (Table S2) were ordered from Life Technologies (Saint-Aubin, France). The PCR assays were run according to the "rapid" program in an ABI cycler.

Statistics

Between-session variation in replicate experiments was corrected using "Factor Correction" [29]. All quantitative values are presented as means ± SEM. Statistical differences for the amino acids fluxes between genotypes were based on homogeneity of variances between the blood sources per genotype followed by one-way analysis of variances using known means and standard deviations. P-values were calculated through multiple comparisons [30].

Results

Characterization of Ass^{fl} mice

To study the function of ASS in the enterocytes of the small intestine, we generated a conditional *Ass* knockout, in which the critical exon 13 is flanked by *loxP* sites (Figure S1). Mice with $Ass^{-/fl}$ and $Ass^{fl/fl}$ genotypes were healthy and fertile. In agreement, Figure 1A shows that flanking exon 13 with *loxP* sites did not affect ASS protein content in the liver, an organ with very high *Ass* expression (Figure 1B). To test if deletion of exon 13 in the mouse was as effective (lethal) as deletion of exon 4 [22], we interbred $Ass^{+/-}$ mice (generated by crossing $Ass^{+/fl}$ mice with deleter-Cre mice [25]). Figure S2A demonstrates that none of the *Ass* exon13-deficient $Ass^{-/-}$ neonates survived longer than 1 day after birth, as was found for the constitutive *Ass* exon4-deficient mice [22]. Together, these data show that mice harboring the Ass^{fl} allele are a good model to investigate the effects of tissue-specific *Ass* deletions. $Ass^{fl/fl}$/$VilCre^{-/-}$ mice are indicated as *Ass*-Con and $Ass^{fl/fl}$/$VilCre^{tg/-}$ as *Ass*-KO/I.

VilCre efficiently and specifically eliminates Ass in the enterocytes

To evaluate the development of the small intestine in the absence of *Ass* expression in the enterocytes, sections of the intestines of ND14, ND18, and ND21 mice were stained with haematoxylin and eosin or with Alcian blue and Nuclear Fast Red. No qualitative differences in the architecture of the duodenum and ileum were found between *Ass*-Con and *Ass*-KO/I mice at any of the ages investigated (not shown). Staining the duodenum and the ileum for the presence of ASS revealed a complete elimination of ASS in the enterocytes of *Ass*-KO/I mice only (Figure 2A–H), whereas ASS in the enteric ganglia remained unaffected. Although both *Ass* and *Vil* are also expressed in the proximal tubules of the kidney [31,32], the *VilCre* transgene is not [23]. Accordingly, we did not observe the disappearance of ASS protein in the kidney (Figure 1B Figure 2I,J). Both *Ass* and *Vil* are also expressed in the epidermis [33,34]. Although ASS protein content in the skin of $Ass^{fl/fl}$/$VilCre^{tg/-}$ mice was lower than in $Ass^{fl/fl}$/$VilCre^{-/-}$ mice (Figure 1B), the difference was not significant.

Development in the absence of Ass expression in the enterocytes

F/A2 mice, which have no net production of arginine in their enterocytes due to the transgenic over-expression of *arginase1* in these cells, suffer from impaired hair and muscle growth and underdevelopment of Peyer's patches due to impaired B-cell development during the suckling period [15,16]. Despite the complete elimination of *Ass* expression in their enterocytes, *Ass*-KO/I mice show a normal increase in body weight (Figure S2B), hair growth (Figure S2C), and development of Peyer's patches (Figure S2D).

Changes in plasma amino-acid concentrations in the absence of Ass expression in enterocytes

Circulating amino acids in arterial, portal, hepatic and renal blood of *Ass*-Con and *Ass*-KO/I mice were determined at ND14 and ND35 (Table S3), with those of arginine, citrulline, ornithine and lysine in ND14 shown in Figure 3A–D. Lysine was included, because it is, like arginine, positively charged and transported by the same transporters, so that differences in the prevalence of arginine would probably be reflected in

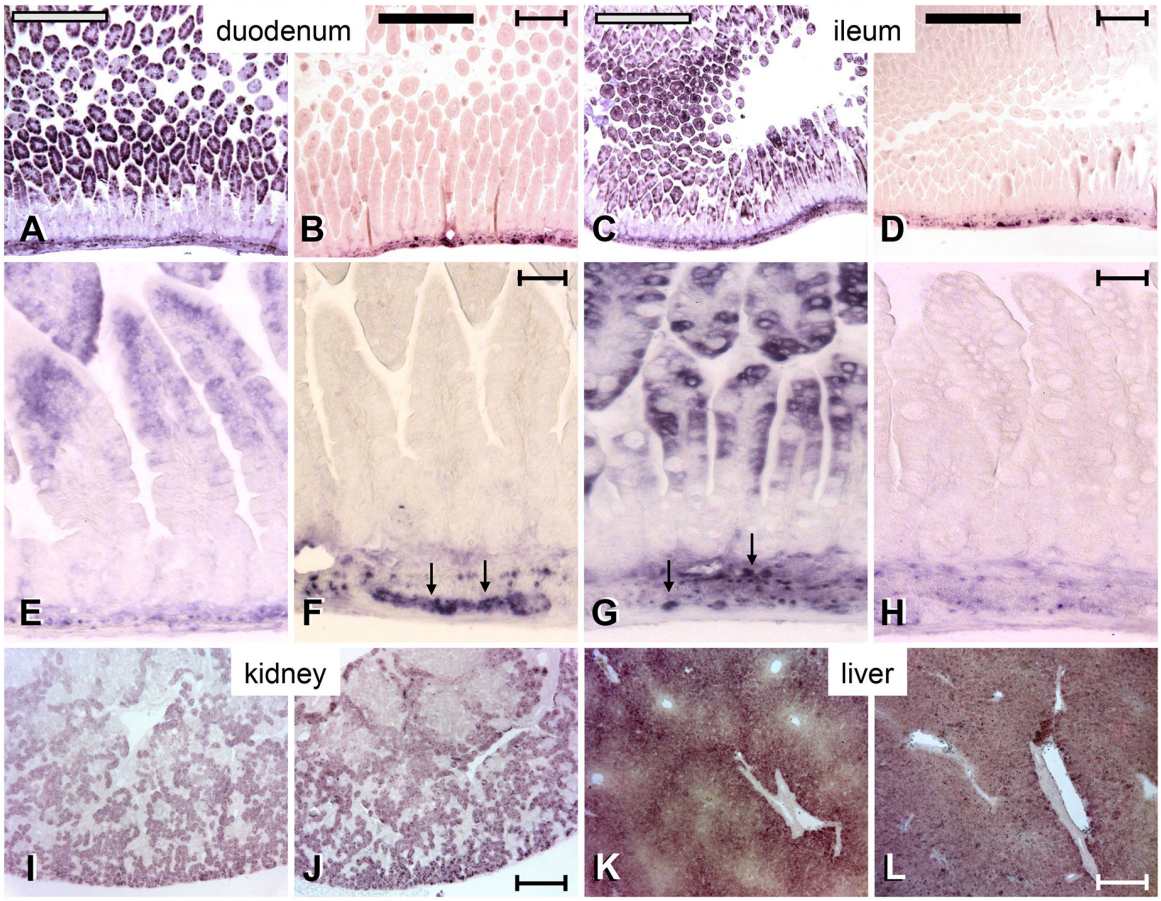

Figure 2. Expression of ASS in the small intestine, kidney and liver of ND14 mice. ASS protein in the duodenum (**panels A, B, E, F**) and ileum (**panels C, D, G, H**) of *Ass*-Con or *Ass*-KO/I animals is visualized by semiquantitative immunohistochemistry. The second row of panels shows magnifications of the top row. Note that expression of ASS in *Ass*-Con mice is limited to the top portion of the villus. Further note that the elimination of *Ass* expression is complete in the enterocytes of *Ass*-KO/I mice, but unaffected in their enteric ganglia (arrows). The bottom panels show unperturbed ASS protein in the S3 segment of the proximal tubule of the kidney (J, K) and in the periportal region of the liver (L, M) of *Ass*-KO/I mice. Gray bars identify *Ass*-Con and black bars *Ass*-KO/I mice. Bars: 8 µm (overviews) and 1 µm (magnifications).

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those of lysine [3536.–37]. Compared to *Ass*-Con mice, the circulating concentration of arginine in plasma of *Ass*-KO/I mice did not change significantly in the aorta and portal vein, but decreased to ~65% in the hepatic vein and increased ~35% in the renal vein (Figure 3A). Circulating concentrations of the arginine precursor citrulline changed more profoundly, being ~1.5-fold higher in the aorta and hepatic vein and ~2-fold higher in the portal and renal veins of *Ass*-KO/I than of *Ass*-Con mice (Figures 3B and 4D). The concentration of ornithine was decreased in the hepatic vein and increased in the renal vein of *Ass*-KO/I compared to *Ass*-Con mice (Figure 3C). Plasma lysine concentrations were not different between control and experimental animals (Figure 3D). With respect to the other amino acids and compared to *Ass*-Con mice, glutamate, alanine and taurine increased in the aorta, glutamate, glutamine, threonine, alanine, taurine, valine, and leucine increased in the portal vein, and alanine, taurine, valine, isoleucine, leucine in the renal vein of *Ass*-KO/I mice, whereas glutamate, valine, isoleucine, tryptophan, and leucine decreased in the hepatic vein (Table S3). In agreement with these findings, the sum of amino acids was higher in the aorta, and portal and renal veins, and lower in the hepatic vein of *Ass*-KO/I than of *Ass*-Con mice. Strikingly, the data of the experimental group were more heterogeneous than those of the control group.

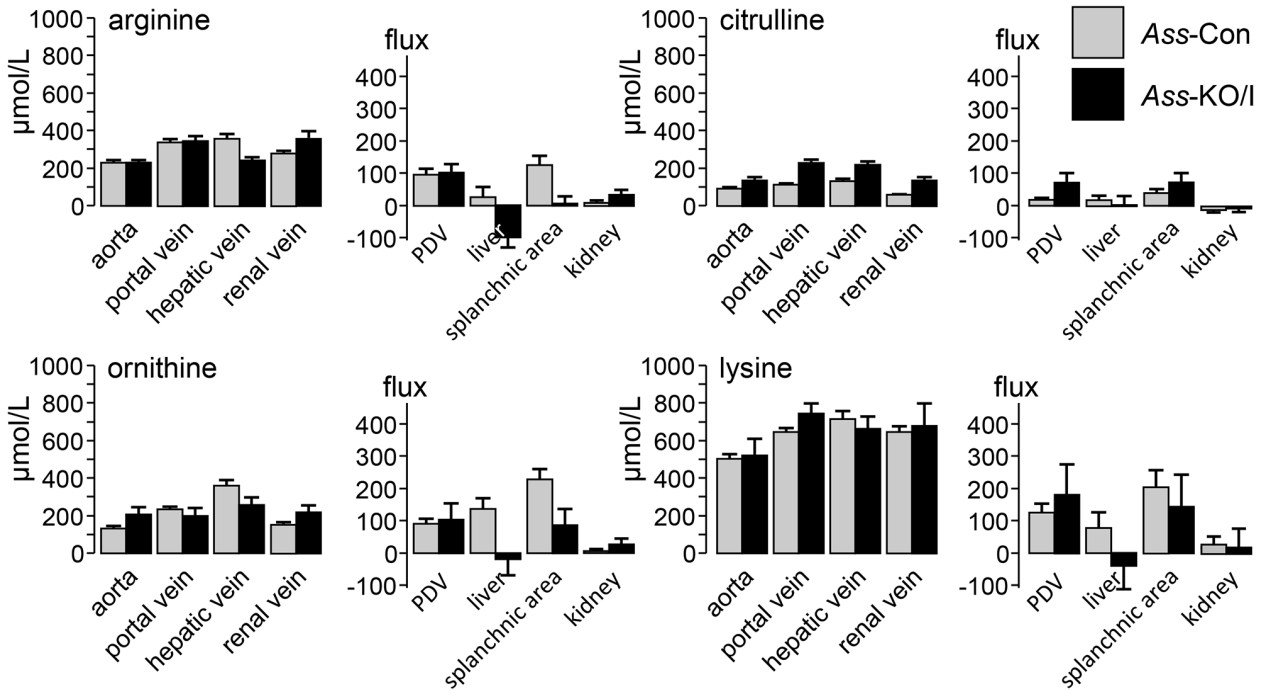

Figure 3. Circulating levels and fluxes of arginine, citrulline, ornithine, and lysine in ND14 mice. Left panels: Circulating concentrations of the indicated amino acids in *Ass*-Con (light gray) and *Ass*-KO/I (black) mice. The source of the blood is indicated. **Right panels**: Production or consumption of the indicated amino acids across the splanchnic region, portal-drained viscera (PDV) and liver.

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Changes in amino-acid fluxes in the absence of ASS in enterocytes

The uptake or release of amino acids across the intestine, liver and kidneys of suckling ND14 mice was determined by multiplying the veno-arterial concentration differences and the blood flow across these organs. To measure the blood flow across the organs, we determined the distribution of radioactive microspheres injected into the left ventricular cavity (Figure 4A). Since we were not able to harvest reference samples from the abdominal aorta of these small mice (~8 gram), only the relative distribution of the cardiac output across organs and, hence, the metabolic fluxes in the portal drained viscera, liver, and kidneys could be calculated. At ND14, ~86% of the transhepatic blood flow originated in the portal vein and ~14% in the hepatic artery, while blood flow through the kidneys amounted to ~33% of that through the liver, without significant differences between genotypes. Relative to *Ass*-Con mice, the production of arginine, ornithine, and lysine in the portal-drained viscera had not changed in *Ass*-KO/I mice, but the liver had become an arginine- and lysine-consuming organ (significance at the trend level), so that the contribution of the splanchnic region to systemic arginine even became non-existent (Figure 3A, right panel). Arginine production in the kidney had more than doubled, but this compensated only ~25% of the decrease in arginine production in the splanchnic area. Citrulline was the only amino acid with an increased (~4-fold) production in the portal drained viscera of *Ass*-KO/I mice, due to the absence of ASS (Figure 3B, right panel). The trends of arginine and lysine were followed by the other amino acids: only the production of glycine and taurine decreased significantly in the portal drained viscera, whereas the liver changed from production to uptake for glutamate, glutamine, taurine, valine, isoleucine, leucine, and the sum of all amino acids (Table S3 Figure 4B). Furthermore, a trend with the same effect was seen for serine, threonine, and methionine. These data therefore show that deletion of *Ass* from the enterocytes of the small intestine has little effect on the small intestine itself, but exerts a major effect on the liver, significantly decreasing the hepatic production of 6 amino acids (glutamate, glutamine, taurine, valine, isoleucine, and leucine) and that of 5 more amino acids (serine, threonine, arginine, methionine, and lysine) at the trend level, that is, changing the liver from an amino acid-producing into an amino acid-consuming organ. Due to the relatively small blood flow through the kidneys (~33% of that

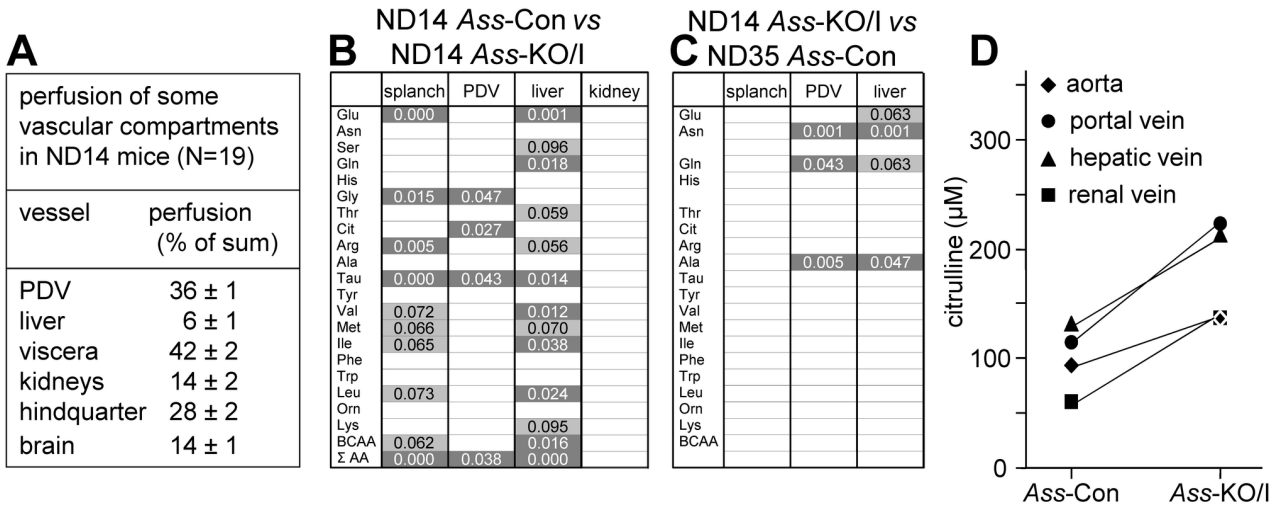

Figure 4. Regional perfusion, differences in amino-acid fluxes and citrulline concentration in ND14 *Ass*-Con and *Ass*-KO/I mice. Panel **A**: Blood flow in portal vein ("Portal Drained Viscera"), hepatic artery (liver), hepatic vein (viscera), renal arteries (kidneys), femoral arteries (hindquarter) and brain as assessed by microsphere distribution after intracardiac injection. Panel **B**: Significant differences in the flux of amino acids across the splanchnic region, portal drained viscera, liver, and kidneys of ND14 *Ass*-KO/I compared to ND14 *Ass*-Con mice. **C**: Significant differences in the flux of amino acids across the splanchnic region, portal drained viscera and liver of ND14 *Ass*-KO/I compared to ND35 *Ass*-Con mice. In panels B and C, dark gray fields represent differences with $P \leq 0.05$. Differences with $0.05 \leq P \leq 0.10$ (light gray) are also shown, because the power of our calculations was weakened by the fact that we could take only one blood sample from a vessel in each ND14 pup. Panel **D**: Average concentration of citrulline in the respective vessels (cf. also Table S3).
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through the splanchnic region), the contribution of the kidney to amino-acid metabolism was still minor at 14 days after birth (Table S3) and not different between controls and knockouts (Figure 4B).

Changes in plasma amino-acid concentrations and fluxes in the absence of ASS in enterocytes

We also measured amino-acid concentrations in arterial, portal and hepatic venous blood of post-weaning (35-days old) mice, that is, when *Ass* is no longer expressed in the small intestine of mice. Table S4A shows that, at this age, *Ass*-KO/I mice no longer differed from *Ass*-Con mice in the concentration of blood amino-acid concentrations. In agreement, the fluxes of amino acids across the portal-drained viscera and liver also differed no longer (Table S4B). Furthermore, it should be noted that, with the exception of glutamine, the liver had become an amino-acid consuming organ in both *Ass*-KO/I and *Ass*-Con mice. Compared to ND14 Ass-KO/I mice, ND35 (Figure 4C) mice merely differed in a further increase in the hepatic consumption of glutamate, asparagine, and alanine, and in the production of glutamine, probably reflecting the detoxification of ammonia in pericentral hepatocytes.

Changes in gene expression in small intestine and liver in the absence of ASS in enterocytes

We designed a PCR array to measure the expression of 45 genes involved in arginine metabolism and arginine, citrulline, or ornithine transport (Table S2). These arrays were tested on 3 small intestines and 3 livers of *Ass*-Con and of *Ass*-KO/I mice. Of these, none except *Ass* differed in intestinal expression between ND14 *Ass*-Con and *Ass*-KO/I mice. In 3 ND14 *Ass*-KO/I livers, however, the expression of *Ass, Asl, Got1, Gpt2, Glud1, Arg1, Arg2, Slc3a2, Slc7a7, Slc7a10, Slc25a13, Slc25a15*, and *Slc38a2* was increased. These results were then confirmed by standard qPCR assays in 5 additional livers of *Ass*-Con and of *Ass*-KO/I mice (Figure 5). In this assay, significant upregulation of gene expression was found for *Asl, Got1, Gpt2, Arg1, Arg2, Slc3a2, Slc25a15*, and *Slc38a2*, demonstrating that the liver adapts to the loss of *Ass* expression in the gut. These 9 genes encode enzymes (*Asl*, *Got1*, *Gpt2*, *Glud1*, *Arg1*, and *Arg2*) and transporters (*Slc25a13* (aspartate/glutamate carrier), *Slc25a15* (ornithine transporter), and *Slc3a2* (light subunit of LAT1 transporter)) that are all directly linked to amino-acid catabolism and urea synthesis.

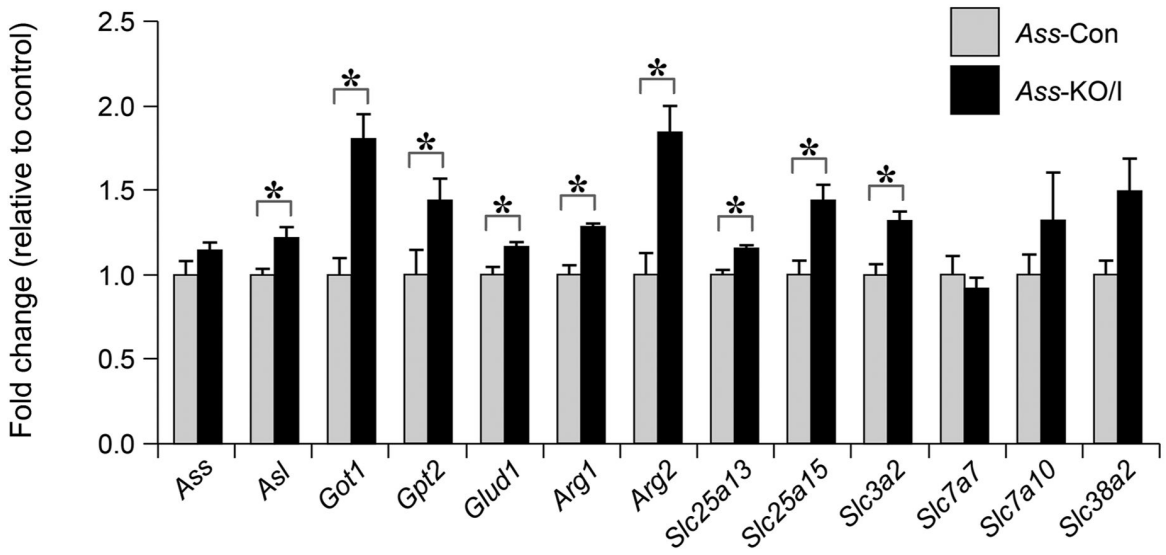

Figure 5. Adaptive changes in arginine transporting or metabolizing genes in the liver of *Ass*-KO/I mice. The 13 genes were selected from a panel of 45 genes (Table S2) and tested on cDNA preparations from 8 *Ass*-Con (light-gray bars) and 8 *Ass*-KO/I mice (black bars). *: P<0.05 between *Ass*-Con and *Ass*-KO/I.
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Discussion

Since milk is relatively deficient in arginine [6–8], endogenous synthesis is necessary to ensure optimal growth and development of neonates during the suckling period. Studies in mice, pigs and humans have identified the enterocytes of the small intestine as a site with a potentially large biosynthetic capacity for arginine that disappears after weaning [9–21,38]. Interestingly, our findings demonstrate that this temporary function of the small intestine is dispensable in mice and compensated for by the liver and, to a lesser extent, the kidneys. Indeed, the genetic ablation of *Ass* from enterocytes did not depress the arterial concentration of arginine. Whereas the change in intestinal function was not associated with a change in gene expression, that of the liver was mediated by a wide-spread upregulation of the expression of genes involved in amino-acid metabolism and transport.

Comparison of Ass-KO/I mice with other arginine-deficient mouse models

Elimination of *Ass* exon 13 by *VilCre*-mediated excision in the small-intestinal enterocytes resulted in undetectable levels of ASS protein in the targeted cells only (Figures 1B and 2). ASS in other tissues, such as the enteric ganglia, kidney tubules, and hepatocytes remained unchanged (Figures 1B and 2). Suckling *Ass*-KO/I mice showed, nevertheless, none of the features of suckling “F/A2” mice, in which intestinal arginine synthesis was annulled by transgenic overexpression of *arginase1* in the enterocytes [15,16]. F/A2 mice suffer from a selective decline in circulating arginine, deficient hair and muscle growth, and underdevelopment of Peyer’s patches. The discrepancy of the phenotypes of *Ass*-KO/I and F/A2 mice suggests that overexpression of *arginase1* causes the gut to function additionally as a sink for circulating and dietary arginine that passes the intestine. Clearly, the capacity of this sink exceeded the endogenous arginine production. The absolute requirement for arginine supplementation of neonatal mice that are constitutively deficient for *ornithine aminotransferase* (*Oat*) [39] also contrasts with the absence of a phenotype in *Ass*-KO/I mice. OAT converts glutamate to ornithine, is expressed at ~5-fold higher levels in the suckling than the weaned small intestine and results in a severe deficiency of ornithine, citrulline and arginine if absent in the neonatal period [39]. Similarly, the *Spf* mutation in ornithine transcarbamoylase (Otc), which severely decreases its capacity to convert ornithine into citrulline, causes a deficiency in circulating citrulline and arginine levels in suckling mice [40]. In aggregate, these mouse models demonstrate that intestinal citrulline is crucial to ensure optimal development whereas intestinal arginine production is, against expectations, expendable to maintain normal circulating arginine levels prior to weaning.

The metabolic consequences of intestinal deletion of Ass

The absence of hypoargininemia or any other detectable sign of arginine-dependent deficiency, therefore, disproved our hypothesis that intestinal arginine synthesis was necessary to supplement endogenous and dietary arginine sources to sustain growth in the suckling. Only for citrulline, we found a significant ~1.8-fold increase in the circulating concentration in *Ass*-KO/I mice. The assessment of the fluxes of amino acids across the portal drained viscera, liver and kidney in the suckling mice (Table S3B) was more laborious than that in adult mice, because only a single vessel could be cannulated per mouse. Furthermore, we could only determine the relative contribution of the intestine and the liver to splanchnic perfusion, because it was not possible to take a reference sample in the suckling mice to determine cardiac output. Our measurements of the blood flow showed, nevertheless, that the contribution of the hepatic artery to liver perfusion (~14%) was similar to that found in adult mice ([41]; Hakvoort & Lamers, unpublished observations). Furthermore, the perfusion of the ND14 kidney was, relative to adult mice, still small (30-35% instead of 70-90% of the total hepatic blood flow ([41]; Hakvoort & Lamers, unpublished). The contribution of the hepatic artery to liver perfusion in mice is, therefore, only ~50% of that in humans. Apart from a ~4-fold increase in citrulline and similarly pronounced decreases in glycine and taurine production, none of the amino-acid fluxes across the portal drained viscera changed, but the flux of 11 individual amino acids and the sum of all amino acids across the liver all changed from production to consumption. It should be emphasized that it is the veno-arterial concentration difference of metabolites and not the blood flow that determines whether an organ produces (positive difference) or consumes (negative difference) metabolites. Despite the sizable concentration of ASS in the kidneys, these organs did not contribute to the metabolic adaptation of *Ass*-KO/I mice. These findings made us investigate the liver as the organ that compensated for the lack of *Ass* expression in the enterocytes.

The liver exerts its homeostatic function with adaptive changes in gene expression

Whereas no changes in gene expression could be detected in suckling intestines from which the capacity to produce arginine had been removed, extensive adaptive changes in gene expression were concurrently induced in the liver, which resulted in the maintenance of a normal plasma arginine concentration in the systemic circulation. Such a homeostatic function of the liver with respect to circulating arginine concentrations was also noticed in metabolic studies of suckling and adolescent pigs. In suckling piglets, plasma arginine concentrations remained similar irrespective of whether they were fed via the gut, the portal vein or the caval vein, even though endogenous arginine synthesis is high only in enterically fed animals [42]. Similarly, arginine-enriched foods, such as soy, increased the concentration of arginine in the portal vein of 2-months old pigs, but this dietary “excess” was neutralized by uptake in the liver [43]. In fact, the switch of liver function from that of amino-acid production to that of amino-acid consumption decreased the net production of amino-acids in the splanchnic region of fed suckling *Ass*-KO/I mice to ~35% of that of their control littermates. Our data show that this switch in liver function was mediated, among others, by widespread increases in expression of arginine-metabolizing and transporting genes. Although the signals that mediate these changes in gene expression in the liver remain to be identified, it is of note that the function of the liver of ND14 *Ass*-KO/I and ND35 *Ass*-Con mice was remarkably similar in the sense that the liver was an amino-acid consuming organ in the absence of arginine biosynthesis in the intestine. The major differences in hepatic function between ND14 *Ass*-KO/I and ND35 *Ass*-Con mice were that the consumption of 3 amino acids, of which alanine was quantitatively the most important, was higher in ND35 mice, and that the production of glutamine had increased. In all likelihood, hepatic glutamine production after weaning reflects the detoxification of ammonia in pericentral hepatocytes. This finding implies that the elimination of arginine synthesis from enterocytes, as occurs naturally after weaning, also induces a post-weaning pattern of amino-acid metabolism in the liver if this capacity is eliminated genetically before birth.

Function of intestinal arginine biosynthesis in neonatal mammals

Although the analysis of genetic mouse models demonstrated that intestinal citrulline synthesis is necessary to maintain normal circulating arginine levels during the suckling period, studies in piglets [20] and (premature) infants [44,45] show that total parenteral nutrition, a condition that is associated with a pronounced decrease in intestinal arginine synthesis in suckling piglets [20], is associated with hypoargininemia and hyperammonemia. These data indicate that intestinal arginine synthesis has a function in piglets and (premature) infants. Extra arginine stimulates, for instance, the synthesis of N-acetylglutamate, a required co-factor for carbamoylphosphate synthetase-1, in enterocytes and hepatocytes [46]. In agreement, some polymorphisms in the carbamoylphosphate synthetase (*Cps1*) gene [47] and hypoargininemia [48,49] predispose premature infants to the development of necrotizing enterocolitis. Similarly, parenteral feeding of premature piglets, which annuls enteral arginine synthesis [9–21,38,42], sensitizes them to a condition that

closely resembles necrotizing enterocolitis [50]. To the best of our knowledge, however, a condition resembling necrotizing enterocolitis is not yet described in neonatal mice. In fact, even though the circulating arginine concentration in neonatal F/A2 mice was only ~30% of control values [15,16,51], we never observed any sign of ischemic lesions in their intestines. Apparently, enteric arginine synthesis is less vital to rodents, possibly because neonatal mice and rats hardly produce urea [52].

Supporting Information

Figure S1. Production and molecular analysis of conditional *Ass* knockout mice. Panel **A**: **S**chematic representation of the *Ass*-targeting construct (AscI – PmeI). The AscI and PmeI sites flanking the construct (14.38 Kb) were introduced by PCR. The XmnI and EcoRI sites upstream from the recombination arms of the targeting construct were used for Southern-blot characterization. Panel **B**: Identification of successfully targeted ES cells. Top subpanel: PCR amplification of vector sequences downstream of AscI (left) or upstream of PmeI (right) identify clones that carried random integrations of the targeting vector. Middle subpanel: Long PCR to demonstrate homologous recombination on 3' side with primer in loxP site and primer downstream of PmeI, yielding a band of 3.7kb. Bottom subpanel: Southern blot of ES cell DNA with proper 3' homologous recombination after digestion with XmnI and EcoRI. Two fragments of the expected size (8.663kb and 10.158kb) were detected with the internal Neo probe. Panel **C**: Wild-type (Ass^{+}) exon-13 allele, floxed (Ass^{fl}) allele, and *Ass*-deficient (Ass^{-}) allele, together with remnant *frt* and *loxP* sites. F1, F2, and R1 indicate the position of the primers used to identify these genotypes (for sequences, see Table S1). Panel **D**: Mouse genotyping. Top subpanel: The F1 and R1 primer pair yield a band of 360 bp for the Ass^{+} allele and 390 bp for the Ass^{fl} allele. Middle subpanel: The F2 and R1 primer pair yield a band of 340 bp for the Ass^{-} allele. Bottom subpanel: The presence of the *VilCre* construct was detected with primer pair Vil-F and Vil-R, giving a band of ~1,100 bp.

Figure S2. Biological effects of deletion of exon 13 of the mouse *Ass* gene. Panel **A**: Cre-mediated homozygous germ-line elimination of exon 13 causes neonatal death, as was previously shown for constitutive elimination of exon 4 [44,45]. **Panels B–D**: *Ass*-KO/I mice grow normally during the first 4 postnatal weeks (B, N = 8 for the wild type and the knockout mice), have normal hair growth (ND14; C) and normal development of Peyer's patches in the small intestine (ND14; D). Light gray bars indicate *Ass*-Con and black symbols *Ass*-KO/I mice.

Table S1. Primer sequences, annealing temperatures and expected PCR-product lengths.

Table S2. Genes tested for expression in the small intestine and liver of ND14 *Ass*-Con and *Ass*-KO/I mice.

Table S3. Plasma amino-acid concentrations and fluxes in ND14 Ass-Con and Ass-KO/I mice. Panel A: Amino acid concentrations in plasma (μM; mean ± SEM). Panel **B**: Amino-acid fluxes (mean ± SEM) of Ass-Con and Ass-KO/I mice are expressed in arbitrary units (arterio-venous difference in concentration * relative flow across the respective organs). Production is indicated in black and consumption in red numbers. Statistical evaluation was performed by ANOVA (see Figure 4B).

Table S4. Plasma amino-acid concentrations and fluxes in ND35 Ass-Con and Ass-KO/I mice. Panel **A**: Amino acid concentrations in plasma (μM; mean ± SEM). Panel **B**: Amino-acid fluxes (mean ± SEM) of Ass-Con and Ass-KO/I mice are expressed in arbitrary units (arterio-venous difference in concentration * relative flow across the respective organs). Production is indicated in black and consumption in red numbers. Statistical evaluation was performed by ANOVA. At this age, no significant differences in intestinal and hepatic amino-acid metabolism were observed between Ass-Con and Ass-KO/I mice (all $P > 0.1$). For a comparison of ND14 Ass-KO/I and ND35 Ass-Con, see Figure 4C.

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Author Contributions

Conceived and designed the experiments: WHL ESK. Performed the experiments: VM SS CdT PvD TBH. Analyzed the data: VM TH ESK. Wrote the manuscript: WHL VM ESK.

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CHAPTER VI

ARGININE DEFICIENCY CAUSES RUNTING IN THE SUCKLING PERIOD BY SELECTIVELY ACTIVATING THE STRESS KINASE GCN2

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Arginine Deficiency Causes Runting in the Suckling Period by Selectively Activating the Stress Kinase GCN2*[S]

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Suckling "F/A2" mice, which overexpress arginase-I in their enterocytes, develop a syndrome (hypoargininemia, reduced hair and muscle growth, impaired B-cell maturation) that resembles IGF1 deficiency. The syndrome may result from an impaired function of the GH-IGF1 axis, activation of the stress-kinase GCN2, and/or blocking of the mTORC1-signaling pathway. Arginine deficiency inhibited GH secretion and decreased liver *Igf1* mRNA and plasma IGF1 concentration, but did not change muscle IGF1 concentration. GH supplementation induced *Igf1* mRNA synthesis, but did not restore growth, ruling out direct involvement of the GH-IGF1 axis. In C2C12 muscle cells, arginine withdrawal activated GCN2 signaling, without impacting mTORC1 signaling. In F/A2 mice, the reduction of plasma and tissue arginine concentrations to ~25% of wild-type values activated GCN2 signaling, but mTORC1-mediated signaling remained unaffected. *Gcn2*-deficient F/A2 mice suffered from hypoglycemia and died shortly after birth. Because common targets of all stress kinases (eIF2α phosphorylation, *Chop* mRNA expression) were not increased in these mice, the effects of arginine deficiency were solely mediated by GCN2.

Arginine is a substrate for the synthesis of proteins, creatine, agmatine, ornithine, and nitric oxide (1). In addition, it functions as a secretagogue for hormones, such as growth hormone (GH) and insulin (2–4). Under normal conditions, endogenous arginine synthesis in adult mammals suffices to sustain daily requirements (5), but a dietary source of arginine may become necessary when demand increases under anabolic or catabolic conditions (5). For this reason, arginine is considered a conditionally essential amino acid.

Arginine deficiency represents a significant metabolic problem in premature infants (6), but its cause remains unknown. In rapidly growing suckling rodents, endogenous arginine biosynthesis is crucial to compensate for the insufficient supply of arginine via the milk (7). The intestine rather than the kidney is primarily responsible for endogenous arginine synthesis in suckling piglets (8, 9). In suckling rodents, the enterocytes of the small intestine also express all enzymes necessary to synthesize arginine (10–13), while arginase is not expressed, thus allowing efficient *de novo* arginine biosynthesis. To examine the function of intestinal arginine synthesis, a transgenic mouse model that overexpresses arginase-I in the enterocytes of the small intestine was produced (14–17). During the suckling period, these "F/A2"[4] mice suffer from a selective deficiency of circulating arginine (which declines to ~25% of controls), reduced growth of hair and skeletal muscle, and inhibition of B-cell maturation (14). The phenotype can be rescued with arginine supplementation (14), but could not be ascribed to a deficiency of arginine metabolites, such as creatine or polyamines, or to arginine-dependent ADP-ribosylation of proteins (14–17). Furthermore, the features of the "arginine-deficiency syndrome" are not seen in mice that are deficient in all three nitric-oxide synthases (18). This leaves two functions of arginine as likely candidates to account for the highly characteristic phenotype of arginine deficiency in suckling mice, viz. its role as a building block in protein synthesis and as a secretagogue of hormones.

Amino acids, including arginine, control translation by two well-established mechanisms. Whenever the intracellular concentration of free arginine decreases below ~20 μM, the charging process of its corresponding tRNA slows down and the concentration of uncharged tRNA increases (19). Uncharged tRNAs activate the ubiquitously expressed "general control

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2 and Figs. S1–S7.
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[4] The abbreviations used are: F/A2, mice overexpressing arginase-I under control of the *Fabp*$_i$ promoter; ATF4, activating transcription factor 4 (*Atf4*); Atrogin1, muscle atrophy F-box E3 ubiquitin-protein ligase or F-box-only protein 32 (*Fbxo32*); CAT-1, cationic amino-acid transporter-1 or Solute carrier 7A1 (*Slc7A1*); CHOP, C/EBP-homologous protein or growth arrest and DNA damage-inducible protein 135 or DNA-damage inducible transcript 3 (*Ddit3*); 4EBP1, eukaryotic translation initiation factor (eIF)4E-binding protein-1 (*Eif4ebp1*); GADD34, growth arrest and DNA damage-inducible protein *34* or protein phosphatase-1 regulatory subunit 15A (*Ppp1r15a*); GCN2, general control non-derepressible 2 kinase or eukaryotic translation initiation factor 2α kinase 4 (*Eif2ak4*); IGF1, insulin-like growth factor 1 (*Igf1*); CKM, muscle-specific creatine kinase (*Ckm*); mTORC1, mammalian target of rapamycin complex-1; MURF1, muscle-specific RING finger-1 or E3 ubiquitin-protein ligase (*Trim63*); MyHC1, myosin heavy chain-I or heavy polypeptide 7 (*Myh7*); MyHC2B, myosin heavy chain-IIb or heavy polypeptide 4 (*Myh4*); ND, neonatal day; S6K1, p70 ribosomal protein S6 kinase-1 or polypeptide 5 (*Rps6ka5*).

non-derepressible2" (GCN2) stress kinase, which then phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF2α) and thereby initiates the "integrated stress response" (20–22). This response blocks cap-dependent protein synthesis, facilitates the translation of specific mRNAs, such as *Atf4* and *Cat-1*, from internal ribosome entry sites, and increases expression of specific transcription factors such as ATF2, ATF4, and CHOP (23), which, in turn, induce the transcription of genes necessary for amino acid synthesis and transport. The other mechanism senses, instead, increases in amino acid concentration, probably via the Rag GTPase complex (24), and results in the activation of the mTORC1 kinase. mTORC1, in turn, regulates cap-dependent translation through the phosphorylation of 4EBP1 and S6K1 (25, 26). The essential amino acid leucine is the prototypic regulator of both branches of amino acid-dependent translational regulation (22, 27). However, for a non-essential amino acid, arginine has an unexpectedly strong effect on mTORC1-dependent signaling (28) and quantitatively rivals leucine *in vitro* (29, 30). The F/A2 phenotype could, therefore, be mediated by activation of GCN2 signaling and/or inhibition of mTORC1-mediated signaling.

The more remarkable phenotypic features of F/A2 mice, namely impaired muscle and hair growth and impaired B-cell maturation, are all also affected by insulin-like growth factor-1 (IGF1) signaling (31–35). Like amino acids, growth factor signals, including those arising from IGF1, stimulate protein synthesis via the mTORC1 pathway (36). The production of IGF1, in turn, is largely dependent on growth-hormone (GH) signaling (37). Arginine, finally, is also a potent growth-hormone secretagogue (38). Arginine deficiency could, therefore, also cause insufficient signaling via the mTORC1 pathway due to an inactive somatotropic axis.

In the present study, we used a combination of *in vivo* and *in vitro* approaches to test the hypotheses that arginine deficiency activates GCN2 signaling or blocks mTORC1 signaling, or both. Our findings demonstrate that arginine deficiency in the neonatal and suckling period activates the GCN2 stress-kinase pathway, without affecting the mTORC1 pathway.

EXPERIMENTAL PROCEDURES

Transgenic Mouse Lines and Animal Husbandry—Transgenic mice that overexpress arginase-I in their small-intestinal enterocytes (F/A2 line (14)) were bred hemizygously. *Gcn2*$^{-/-}$ mice (39) originated from the colony of D. Ron (New York, NY). Control animals were littermates of the experimental animals. Animal studies were reviewed and approved by the committee for animal care and use of Maastricht University.

GH Administration—Litters were limited to 5 animals on neonatal day 1 (ND1) and weighed daily. Starting on ND3, 3 mg/kg body weight of human recombinant growth hormone (hrGH; Humatrope, Lilly, Indianapolis, IN) was administered subcutaneously in 25 μl of vehicle twice daily (40). Control animals were injected with vehicle alone.

Blood and Tissue Collection—Animals were sacrificed by decapitation. Blood was collected into heparin-containing tubes and centrifuged at 2,000 $\times$ *g* for 5 min at 4 °C. IGF-1 protein concentrations were measured in plasma, whereas amino acids were measured in deproteinized plasma. 80 μl of plasma was added to 6.4 mg of lyophilized sulfosalicylic acid, vortexed, and stored at −20 °C. For amino acid measurements, the tissues were homogenized with silica mini-beads in 0.33 M sulfosalicylic acid. Tissues were collected at ND0, ND10, ND17, and ND21. Amino acid concentrations were determined by HPLC (41). Tissues for protein and mRNA analysis were isolated, snap frozen in liquid nitrogen and stored at −80 °C. For histological analysis, tissues were fixed in 4% buffered formalin and embedded in paraffin.

IGF-1 Assay—Mouse IGF-1 concentration in plasma and muscle was measured by the m/r IGF-1 E25 enzyme immunoassay, as detailed by the manufacturer (Mediagnost, Reutlingen, Germany). This assay eliminates IGFBP interference with the IGF-1 measurement.

RNA Extraction and Quantitative PCR—Total RNA was extracted with Trizol (Sigma). 1 μg of total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). Primer sequences (supplemental Table S1) were optimized for an annealing temperature of 60 °C, except for the *Igf1* primers that were annealed at 70 °C. cDNA samples for mRNA determination were diluted 60-fold before use and those for 18 S rRNA 200-fold. Primary fluorescent data were exported and analyzed with the Lin-Reg Analysis program (42). If reverse transcriptase was omitted, no product formed. mRNA concentrations were expressed relative to 18 S rRNA content. Between session variations in replicate experiments were corrected using factor correction (43).

Western Blots, Immunostaining, and Immunofluorescence—For Western blot assays, 100 μg of protein were loaded per lane. Antibody (supplemental Table S2) binding was visualized using the Super Signal West Femto Maximum Sensitivity Substrate (Pierce). For detection of the phosphorylated and unphosphorylated forms of eIF2α, the Odyssey Infrared Imaging system (Li-cor Bioscience, UK) was used.

For immunostaining, 5-μm sections of tissues were prepared. After deparaffinization, sections were preincubated with Teng-T (10 mM Tris (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20)/10% normal goat serum (NGS) for 30 min, followed by an overnight incubation with the primary antibody diluted in Teng-T/10% NGS. Slides were then washed with PBS and incubated with Teng-T/10% NGS for 15 min, followed by 90 min incubation with the secondary antibody (supplemental Table S2) in Teng-T/10% NGS. After washing, the slides were incubated with alkaline phosphatase substrate (NBT/BCIP tablets; Roche) at 37 °C. Digital images of the sections were analyzed with the Leica Quantimet 500 Image Analysis System, v3.2 (44).

Statistical Analysis—The significant difference for one variable between two distinct groups was analyzed using an unpaired Student's *t* test. For more complex analyses of significance for variables with different controls, each gene was analyzed using Gaussian linear regression, including 18 S as housekeeping gene. When appropriate, additional explanatory variables such as tissue type, strain, and time were included in the model. Finally, interactions among the included variables were considered. The inference criterion used for comparing the models is their ability to predict the observed data, *i.e.* models are compared directly through their minimized minus log-

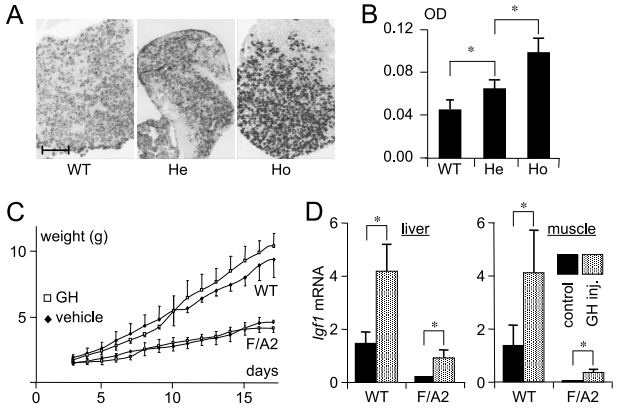

FIGURE 1. **Role of growth hormone in the phenotype of F/A2 mice.** *Panels A* and *B*: Detection of GH in pituitary somatotropes of ND21 mice. *A*, sections of pituitary glands of ND21 WT, hemizygous F/A2 mice (*He*), and homozygous F/A2 mice (*Ho*) were stained for the presence of GH. Bar: 5 μm. *B*, optical density (*OD*) in GH-positive cells ($n = 3$ mice per genotype) increases from wild-type via hemizygous to homozygous F/A2 mice. *Panels C* and *D*, effect of GH treatment on weight gain in suckling F/A2 mice. *C*, change in body weight of F/A2 mice and their wild-type littermates during treatment with GH (twice daily 3 mg rhGH/kg body weight subcutaneously) between ND3 and ND21 ($n = 6$ mice per genotype). Note that F/A2 mice show a ~3-fold lower growth rate compared with wild-type mice. *D*, quantification of *Igf1* mRNA in liver (*left*) and muscle (*right*) of vehicle and GH-injected ND17 mice. $n = 3$ mice per genotype. *, $p < 0.01$.

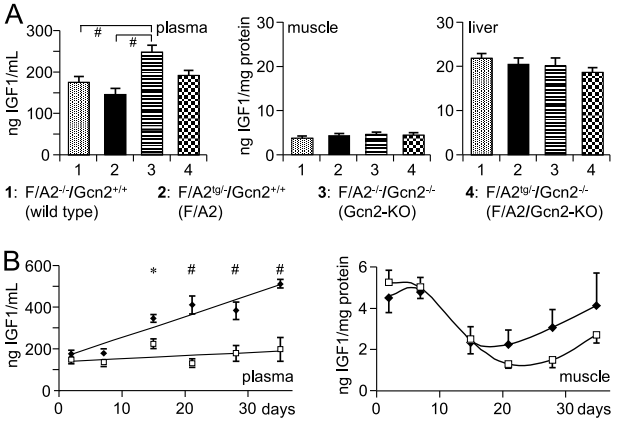

FIGURE 2. **Plasma and tissue IGF1 protein concentrations in F/A2 mice.** *Panel A*, differences in plasma, muscle and liver IGF1 protein concentration in ND1 $F/A2^{-/-}/Gcn2^{+/+}$ (wild-type), $F/A2^{tg/-}/Gcn2^{+/+}$ (F/A2 transgenic), $F/A2^{-/-}/Gcn2^{-/-}$ (Gcn2-KO), and $F/A2^{tg/-}/Gcn2^{-/-}$ (F/A2/Gcn2-KO double mutant) mice ($n = 6$ per genotype). The plasma IGF1 concentration in $F/A2^{tg/-}/Gcn2^{-/-}$ tended to be lower than in $F/A2^{-/-}/Gcn2^{-/-}$ mice ($p = 0.055$). *Panel B*, changes in plasma and muscle IGF1 protein concentration during the first 5 postnatal weeks in wild-type (*black diamonds*) and F/A2 mice (*white squares*); ($n = 3$ per genotype except at ND1). *, $p < 0.05$; #, $p < 0.01$. The age-dependent changes in muscle IGF1 content were highly significant ($p < 0.01$).

likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion (45). If the most likely model contains the treatment effect, the fold change representing the ratio between the variable of the treated group and the same variable for the control is computed, as well as its 95% confidence interval.

RESULTS

The GH/IGF1 Axis Is Functional but Repressed in F/A2 Mice—The phenotype of F/A2 mice is compatible with a deficiency of GH and/or IGF1. We, therefore, investigated the GH content in the pituitary gland by quantitative immunohistochemistry (Fig. 1*A*). The somatotropes of 3-week-old hemi- and homozygous F/A2 mice contained 1.4- and 2.2-fold more GH, respectively, than those of their wild-type counterparts (Fig. 1*B*), indicating an inverse correlation with the severity of the F/A2 phenotype (14, 16).

We then used the rat pituitary cell line GH3, which produces GH (46, 47), to assess the direct effects of arginine on GH production and secretion. GH content in the GH3 cells was assessed by quantitative immunohistochemistry (supplemental Fig. S1, *A* and *B*) and ELISA (supplemental Fig. S1*C*). Both assays showed that, after 24 h of culture, the cellular GH content was inversely correlated with extracellular arginine concentration and decreased from ~50 to ~25 pg of GH per cell at 0 and 200 μM arginine in the medium, respectively (supplemental Fig. S1*C*; significant at ≥50 μM arginine). This decrease in cellular GH content was accounted for by a 1.5-fold increase in concentration of GH in the medium (significant at ≥25 μM arginine). Because the total GH content of the cells and the medium combined did not vary with arginine concentration in the medium, these findings reveal a pronounced concentration-dependent effect of arginine on GH secretion. The dependence of GH secretion on the ambient concentration of arginine after 72 h of culture was similar to that observed after 24 h (not shown). These data show that, below a concentration of 50 μM ambient arginine, GH secretion is reduced, with a half-maximal effect at ~20 μM arginine.

As early as 3 days after birth (ND3), F/A2 mice can be distinguished from their wild-type littermates by a lower body weight (14). To assess whether the typically hypomorphic tissues of F/A2 mice (muscle, hair, and B-cells (15)) respond to GH supplementation, mice were subcutaneously injected twice daily with 3 mg/kg recombinant human (rh)GH from ND3 onwards. This dose of rhGH mediates growth in liver IGF-1-deficient mice (40). Fig. 1*C* shows that the growth rate of homozygous F/A2 mice was ~50% of that of wild-type mice and that treatment with rhGH had no effect on the growth rate of either wild-type or F/A2 mice.

GH action is mediated by GH receptors (GHR) and results in the synthesis and secretion of IGF1 in target tissues. *Ghr* mRNA concentrations in the livers of ND1, ND10, and ND21 mice increased with age, but were not different in wild-type and F/A2 mice (supplemental Fig. S2). To assess the functionality of these GHRs and their downstream signaling pathway, *Igf1* mRNA concentrations were quantified in liver and muscle of GH-treated and control mice (Fig. 1*D*). In both tissues, *Igf1* mRNA concentrations in F/A2 mice were only 10–15% of those in wild-type mice. Although the rhGH injections increased *Igf1* mRNA concentration in liver and muscle of both F/A2 and wild-type mice considerably (~8- and ~2.5-fold, respectively; $p < 0.01$), *Igf1* mRNA levels in the liver and muscle of treated F/A2 mice increased to only ~65 and ~25%, respectively, of that in untreated wild-type mice. We then measured plasma and tissue IGF1 concentrations to assess whether F/A2 mice suffer from a low production of IGF1 (Fig. 2). In wild-type and

F/A2 ND1 mice, no differences in plasma, muscle, or liver IGF1 protein were detected (Fig. 2*A*). However, plasma IGF1 levels increased ~7-fold faster in wild-type than F/A2 mice during the first 5 postnatal weeks (Fig. 2*B*, *left panel*; $p < 0.001$)). Plasma IGF1 levels reflect hepatic IGF1 production, but liver IGF1 does not affect growth until 6 weeks postnatally (48, 49). We, therefore, measured IGF1 levels in an affected tissue (calf muscle) of the same mice (Fig. 2*B*, *right panel*). Muscular IGF1 concentration decreased gradually between 1 and 3 weeks after birth, without difference between F/A2 and wild-type mice. After weaning, the IGF1 concentration in muscle increased again, with a ~1 week delay in F/A2 relative to wild-type mice. Although the age-dependent changes in muscle IGF1 content were significant ($p < 0.01$), they did not differ between F/A2 and wild-type mice. Because IGF1 in skeletal muscle affects growth only after the 3rd postnatal week (32), that is, coincident with the resumption of growth in F/A2 mice (14), we further concluded that, although the GH-IGF-1 axis did not function properly in F/A2 mice, its disturbed function could not account for the marked hypotrophy seen in these animals.

Arginine Is Essential for Muscle Development—F/A2 skeletal muscles have an immature appearance (14). We, therefore, investigated if arginine directly affects muscle development in differentiating C2C12 myoblasts that were cultured in the presence of different concentrations of arginine. Supplemental Fig. S3*A* shows representative pictures after 4 days of myogenic differentiation in the presence (200 μM) or absence of arginine. Both the myogenic index (the fraction of nuclei residing in cells with three or more nuclei and considered an early differentiation parameter; supplemental Fig. S3*B*) and the cellular concentration of the late differentiation marker creatine kinase-M (supplemental Fig. S3*C*) increased with increasing arginine concentration to plateau at 25–50 μM arginine. Myogenic differentiation is, therefore, clearly dependent on arginine availability, with a half-maximal effect at 10–20 μM arginine in the medium.

Arginine Deficiency Induces the Integrated Stress Response in Vitro—Amino acid deficiency can activate the GCN2 stress kinase pathway (50). An early step is the phosphorylation of translation factor eIF2α (51). As anticipated, arginine deprivation of C2C12 cells at 80% confluence induced phosphorylation of eIF2α on serine 51 within 30 min. The protein remained phosphorylated for at least 2 h (supplemental Fig. S4*A*). Furthermore, arginine depletion induced the accumulation of the transcription factors ATF4 and CHOP, which are well-established downstream mRNA targets of the GCN2 stress-kinase pathway (23, 52), in a concentration-dependent way in both C2C12 and GH3 cells (supplemental Fig. S4*B*). In both cell types, *Chop* mRNA concentration increased more gradually with declining arginine concentration in the medium than *Atf4* mRNA, indicating that *Chop* mRNA accumulation was more sensitive to arginine removal than that of *Atf4* mRNA. The adaptive response of *Atf4* and *Chop* mRNA expression in primary cultures of mouse B-lymphocytes, another hypomorphic tissue in F/A2 mice, to arginine deprivation was similar to that of C2C12 and GH3 cells (not shown). Supplemental Fig. S4*C*, finally, shows that ATF4 protein accumulates at arginine concentrations below 25 μM (see Ref. 52). These findings reveal that the integrated stress response becomes induced at arginine concentrations in the medium below 50 μM, with an EC_{50} of ~12.5 μM.

Arginine Deficiency Does Not Affect Signaling through the mTORC1 Pathway—Amino acid deficiency can regulate protein synthesis by up-regulating signaling via the GCN2 pathway and by down-regulating signaling via the mTORC1 pathway. We, therefore, compared the effects of 0, 12.5, and 200 μM arginine in the culture medium on the degree of phosphorylation of the translation factors eIF2α as target of GCN2 kinase and 4EBP1, and S6K1 as targets of mTORC1 kinase in the same cell extracts (supplemental Fig. S5, column "Ctrl"). As expected, arginine deficiency caused a significant >2-fold increase in eIF2α phosphorylation, but did not significantly affect either S6K1- or 4EBP1 phosphorylation ($p = 0.13$ and 1.0, respectively). Furthermore, arginine deficiency increased the mRNA concentrations of *Atf4*, ATF4 target *Chop*, and CHOP target *Gadd34*, which dephosphorylates eIF4α-P. In addition, the expression of the ATF4 target *Cat-1* was induced. Collectively, these data show that the ambient arginine concentration affects the activity or expression of all investigated parameters except the mTORC1 targets S6K1 and 4EBP1.

The eIF2αP-specific phosphatase inhibitor salubrinal was used to assess to what extent arginine deficiency induced maximal eIF2α phosphorylation and whether the degree of eIF2α phosphorylation affected the stress-kinase esponse only quantitatively or also qualitatively (supplemental Fig. S5, column "Sal"). At the concentration used (50 μM), salubrinal increased eIF2α phosphorylation ~3-fold over basal levels ($p < 0.001$) and abolished the dependence of eIF2α phosphorylation and its downstream targets on arginine concentration. Furthermore, salubrinal decreased overall S6K1 phosphorylation levels ~3-fold ($p < 0.001$) and increased the mRNAs of *Atf4* (~3-fold; $p < 0.001$), *Chop* (~20-fold; $p < 0.001$), *Gadd34* (~6-fold; $p < 0.001$), and *Cat-1* (~3-fold; $p < 0.001$), that is, the ~3-fold increase in eIF2α phosphorylation had quantitatively a disproportional effect on its downstream targets. These data reveal that the effects of a graded phosphorylation of eIF2α due to arginine deficiency differ both qualitatively and quantitatively from the effects of a maximal phosphorylation due to salubrinal treatment.

Constitutive *Gcn2* knockdown in C2C12 muscle cells by integration of a viral vector carrying *Gcn2*-specific shRNA reduced *Gcn2* mRNA expression to ~25% of control values (not shown). It strongly inhibited myotube formation, suggesting that GCN2 activation is involved in myocyte differentiation. This level of knockdown did, however, not affect the arginine-dependent stress-kinase response (supplemental Fig. S5, column "Gcn2-kd"). Similarly, treatment with the muscle growth factor IGF1 did not mediate, at the concentration used (0.13 nM), a change in effects on the arginine-dependent phosphorylation of eIF2α, S6K1, or 4EBP1 ($p < 0.05$, $p = 0.18$, and $p = 0.66$, respectively) or their downstream targets.

Collectively, these data show that arginine deficiency induced a graded and comparatively mild increase in eIF2α phosphorylation and ATF4-dependent gene expression, but did not affect phosphorylation of S6K1 or 4EBP1. However, when eIF2α became hyperphosphorylated after treatment with

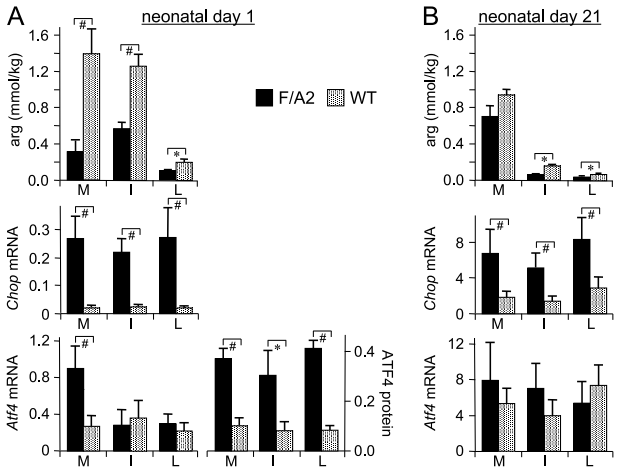

FIGURE 3. **The stress-kinase pathway is activated in neonatal F/A2 mice.** *Panel A*, concentration of arginine, *Chop* mRNA, and *Atf4* mRNA and ATF4 protein in skeletal muscle (*M*), small intestine (*I*), and liver (*L*) of ND1 F/A2 and wild-type mice. mRNA concentrations were corrected for 18 S rRNA content, while equal protein loading was checked by pre-staining Western blots with Ponceau-S. $n = 8–12$ mice per genotype for arginine and mRNA concentration, and $n = 3$ for ATF4 protein. *Panel B*, concentration of arginine, and *Chop* and *Atf4* mRNA concentration in muscle, small intestine, and liver of ND21 F/A2 and wild-type mice. $n = 8–12$ mice per genotype. *, $p < 0.05$; #, $p < 0.01$.

TABLE 1

Distribution of genotypes and plasma glucose concentrations in the offspring of crosses of hemizygous F/A2 *(F/A2$^{tg/-}$)* mice and heterozygous Gcn2 *(Gcn2$^{+/-}$)* mice

Panel A: six different genotypes could be identified by PCR ($F/A2^{tg/-}$ and $F/A2^{tg/tg}$ mice could not be distinguished; $F/A2^{-/-}$ mice denote wild-type animals). A total of 60 mice (dead and alive) were analyzed. Four newborn mice disappeared from the nests in the first two days and could not be analyzed. Panel B: blood was collected from mice at ND1.

		Day 0	Day 2	
Genotype (A)	Expected	Alive	Alive	Dead
$F/A2^{-/-}/Gcn2^{+/+}$	4	0		
$F/A2^{-/-}/Gcn2^{+/-}$	8	10	10	
$F/A2^{-/-}/Gcn2^{-/-}$	4	6	5	1
$F/A2^{tg/?}/Gcn2^{+/+}$	11	10	10	
$F/A2^{tg/?}/Gcn2^{+/-}$	22	23	23	
$F/A2^{tg/?}/Gcn2^{-/-}$	11	7	0	7
$F/A2^{?/?}/Gcn2^{?/?}$		4		4
Total		60	48	12

	Plasma glucose	
Genotype (B)	mM	N
$F/A2^{-/-}/Gcn2^{+/+}$	3.3 ± 0.2	23
$F/A2^{-/-}/Gcn2^{-/-}$	2.8 ± 0.3	7
$F/A2^{tg/?}/Gcn2^{+/+}$	3.2 ± 0.2	25
$F/A2^{tg/?}/Gcn2^{-/-}$	2.7 ± 0.2	15

the phosphatase inhibitor salubrinal, this specificity was lost. *Gcn2* knockdown blocked myotube differentiation, without totally eliminating the dependence of eIF2α phosphorylation on ambient arginine concentrations. The muscle growth factor IGF1, finally, increased the effect of arginine deficiency.

The Stress Kinase Pathway Is Activated in F/A2 Mice—On neonatal day 1, the tissue concentration of arginine in skeletal muscle was only 20% ($p < 0.01$) and that in intestine and liver ~50% ($p < 0.01$ and <0.05, respectively) in F/A2 compared with wild-type mice (Fig. 3*A*, *top subpanel*). The low concentration of arginine in liver can be ascribed to the high concentration of the urea cycle enzyme arginase-1 in the liver. The low tissue arginine concentration induced *Chop* mRNA expression >8-fold in all tissues investigated ($p < 0.001$; Fig. 3*A*, *middle subpanel*), but *Atf4* mRNA expression in skeletal muscle only (~3-fold; $p < 0.05$; Fig. 3*A*, *bottom subpanel*). These findings *in vivo* reveal that the tissue arginine concentration had decreased most in muscle and that *Chop* mRNA concentration was a more sensitive parameter to monitor the stress-kinase response to amino acid deprivation than *Atf4* mRNA.

Because phosphorylation of eIF2α facilitates translation of *Atf4* mRNA (53), we also determined ATF4 protein concentration in ND1 mice (Fig. 3*A*, *bottom subpanel*). Western blot analysis of muscles of F/A2 mice showed the expected 38-kDa band (*bold arrow* in supplemental Fig. S6) and a much weaker band at ~50kDa (*small arrow*), whereas these bands were absent in muscle of wild-type mice. The ~50kDa band may correspond to an ubiquitinated form of ATF4 (54). ATF4 protein was 3–4-fold higher in muscle, small intestine, and liver of F/A2 than of wild-type animals (Fig. 3*A*, *bottom subpanel*; $p < 0.01$, <0.02, and <0.01, respectively). The data in Fig. 3*A* demonstrate that *Chop* mRNA and ATF4 protein are equally sensitive parameters to assess the response to arginine deficiency in muscle, intestine, and liver.

On neonatal day 21, the concentration of arginine in wild-type muscle was similar to that on ND1, but in the intestine and liver, it had decreased to ~15 and ~30% of that on ND1 ($p < 0.01$). This pronounced decline can be ascribed to the cessation of intestinal arginine biosynthesis just prior to weaning (10) and the continued maturation of the function of the urea cycle in the liver (55). The arginine concentration in F/A2 muscle had increased 2-fold relative to that on ND1 ($p < 0.05$) and was no longer different from that in controls (Fig. 3*B*, *top subpanel*). However, the concentration of arginine in F/A2 intestine and liver remained depressed ($p < 0.05$). Tissue *Chop* and *Atf4* mRNA concentration had increased ~20-fold and ~10-fold, respectively, relative to ND1 (Fig. 3*B*, *middle* and *bottom subpanels*). *Chop* mRNA concentrations were still significantly elevated in F/A2 tissues ($p < 0.01$; Fig. 3*B*, *middle subpanel*, but *Atf4* mRNA concentrations in F/A2 mice were no longer different from those in control mice (Fig. 3*B*, *bottom subpanel*). Together, these *in vivo* data show that the stress-kinase response pathway is strongly activated in neonatal F/A2 mice. This activation persists throughout the suckling period, although its severity appears to decline, in agreement with the disappearance of the phenotype after weaning.

GCN2 Mediates the Activation of the Stress Response in Arginine-deficient Neonatal Mice—In addition to GCN2, three other stress kinases can phosphorylate eIF2α. To demonstrate that GCN2 is the only stress kinase that becomes activated in neonatal F/A2 mice, we crossed $F/A2^{tg/-}$ mice with $Gcn2^{-/-}$ mice. Table 1*A* lists the genotypes of the offspring and shows that the respective genotypes were born at the expected Mendelian frequency. This Table also shows that all F/A2 mice that were deficient for *Gcn2* ($F/A2^{tg/?}/Gcn2^{-/-}$) died within 48 h after birth ($p < 0.001$). The plasma arginine concentration in F/A2 neonates was only 25–40% of that in control neonates, irrespective of whether *Gcn2* was or was not expressed ($p < 0.01$; Fig. 4*A*). *Gcn2*-deficient F/A2 neonates did drink, but their plasma glucose concentration on ND1 was significantly lower

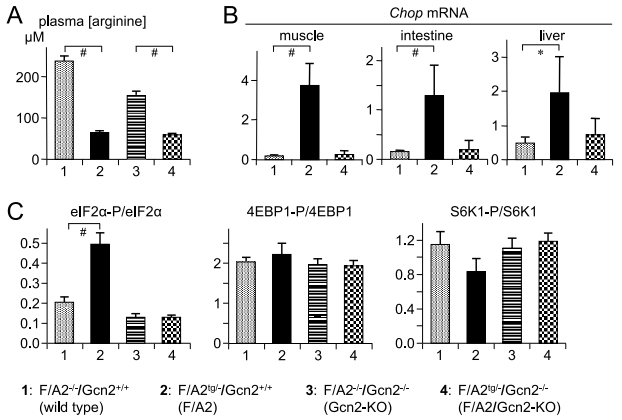

FIGURE 4. **GCN2 is responsible for the induction of the integrated stress response in neonatal F/A2 mice.** *Panel A*, arginine concentration in trunk blood of ND1 mice. *Panel B*, *Chop* mRNA expression in muscle, small intestine, and liver of the same mice as shown in *panel A*. *Panel C*, Ser51-eIF2α, Ser65–4EBP1, and Thr389-S6K1 phosphorylation in skeletal muscle of ND1 mice. n = 8–12 mice per genotype. *, $p < 0.05$; #, $p < 0.01$.

than that in wild-type controls ($p < 0.01$), with *Gcn2*-deficient and F/A2 neonates taking intermediate positions (Table 1*B*).

To establish whether *Gcn2* deficiency prevented the activation of the stress response, we determined *Chop* mRNA concentrations in muscle, small intestine and liver of neonatal wild-type mice ($F/A2^{-/-}/Gcn2^{+/+}$), F/A2 mice ($F/A2^{tg/?}/Gcn2^{+/+}$) and F/A2 mice that were also *Gcn2*-deficient ($F/A2^{tg/?}/Gcn2^{-/-}$) (Fig. 4*B*). As expected, *Chop* mRNA concentrations were low in muscle, liver and gut of wild-type ($F/A2^{-/-}/Gcn2^{+/+}$) mice and elevated in the same organs of F/A2 ($F/A2^{tg/?}/Gcn2^{+/+}$) mice ($p < 0.01$, <0.01, and <0.05, respectively). However, the elevation of *Chop* mRNA concentration was not found in muscle, liver, and gut of the F/A2 neonates that were, in addition, deficient for *Gcn2* ($F/A2^{tg/?}/Gcn2^{-/-}$). Similarly, the degree of phosphorylation of eIF2α in muscle was increased in F/A2 mice ($p < 0.01$), but not in F/A2 mice that were also deficient in *Gcn2* (Fig. 4*C*, *left subpanel*, and supplemental Fig. S6). These findings demonstrate that GCN2 activation is necessary for postnatal survival in F/A2 mice and that the phosphorylation of eIF2α and the induction of *Chop* expression in these mice are solely dependent on GCN2 activation. *Gcn2*-deficient neonates further had a significantly higher plasma IGF1 level than wild-type or F/A2 mice (Fig. 2*A*, *left subpanel*). F/A2 neonates had a lower plasma IGF1 level than wild-type or *Gcn2*-deficient neonates, but the difference was significant for only the latter.

mTORC1-mediated Translational Control Is Not Affected in Arginine-deficient Neonatal Mice—The degree of phosphorylation of S6K1 and 4EBP1 in neonatal skeletal muscle was not significantly different in any of the four genotypes (Fig. 4*C* and supplemental Fig. S6; $p = 0.36$ and 0.71, respectively). These findings indicate that the reduction in circulating arginine concentration in F/A2 mice does not affect signaling through the mTORC1 signaling pathway. *Ckm*, *Myhc1*, and *Myhc2B* mRNA concentrations were not affected either by arginine or *Gcn2* deficiency in neonates (supplemental Fig. S7).

DISCUSSION

Suckling F/A2 mice, which express arginase I from the *Fabp1*-promoter exclusively in the enterocytes of the small intestine (14), suffer from a severe reduction in plasma arginine levels (~70 μM *versus* ~230 μM), reduced hair and muscle growth, and a specific block in B-cell development (14–17). In this study, we show that the deficiency of arginine causes an impaired function of the endocrine GH/IGF1 axis of these mice, but that the compromised function of the somatotropic axis does not account for the observed growth deficiency. Instead, activation of the GCN2-dependent stress response was found to be responsible for the hampered growth and even to be necessary for postnatal survival. Arginine deficiency does not suppress growth factor (mTORC1)-dependent signaling.

The Somatotropic Axis Is Not Responsible for the Runting Phenotype of F/A2 Mice—The gradual accumulation of GH in the pituitary gland from wild-type mice via hemizygous to homozygous F/A2 mice suggests that decreasing circulating concentrations of arginine progressively suppresses GH synthesis or secretion. Using GH3 cells, we could demonstrate that arginine deficiency blocks GH secretion rather than synthesis. Our findings, therefore, complement the recent finding that chronic arginine supplementation stimulates GH secretion (56) and imply that a broad dose-response relation exists between circulating arginine and GH secretion. The severely reduced plasma IGF1 concentration in suckling and weanling F/A2 mice also underscores the presence of a GH deficiency in F/A2 suckling mice, as IGF1 production is dependent upon GH stimulation (38, 57) and >75% of plasma IGF1 is produced by the liver (48, 49). *In vivo*, the effects of arginine deficiency on growth in suckling mice become apparent when the circulating arginine concentration falls below ~80 μM (14), which compares well with our present finding *in vitro* that arginine concentrations below 50 μM inhibit GH secretion (neonatal plasma arginine concentrations are ~1.6-fold higher than those after weaning (14, 16)). However, neonatal F/A2 mice are not growth-retarded, even though their plasma arginine concentration is low, whereas *Igf1*-deficient mice are (58, 59). Furthermore, GH supplementation could not restore growth in suckling F/A2 mice. In addition, IGF1 concentration in skeletal muscle, one of the most severely affected tissues, was similar in F/A2 and wild-type mice. These findings demonstrate that the activity of the somatotropic axis is impaired in arginine-deficient suckling mice, but not directly responsible for the runted phenotype observed in these mice.

Arginine Deficiency Selectively Induces the Integrated Stress Response in Vitro—Because skeletal muscle is a severely affected tissue in F/A2 mice (14), we used the C2C12 muscle cell line to investigate the mechanism underlying the observed effects of arginine deficiency. Whereas arginine withdrawal increased, as expected, eIF2α phosphorylation and the expression of its established downstream targets *Atf4, Chop, Gadd34*, and *Cat-1* (39, 60, 61), it did not significantly affect the degree of phosphorylation of S6K1 or 4EBP1, demonstrating that, *in vitro*, a deficiency of arginine activates the stress kinase GCN2, but does not block mTORC1 signaling. The pronounced effect of arginine deficiency on GCN2 activation contrasts with an

earlier report that the depletion of all essential amino acids for 6 h does not activate the GCN2 pathway in C2C12 myotubes (62). It is well possible that, as the authors themselves suggest (62), the complete removal of all essential amino acids strongly induces intracellular proteolysis in myotubes, whereas the selective depletion of arginine does not, or to a much lesser extent, as shown by the absence of an effect of arginine withdrawal on the expression of the ubiquitin ligases *Atrogin1* and *Murf-1*.[5] These discrepant findings underscore, nevertheless, that results obtained after complete removal of amino acids (*e.g.* Ref. 62), sometimes followed by adding back a single amino acid (30), often differ from results obtained after selectively removing a single amino acid from a complete mixture (29, 63) and present study). We opted to selectively remove arginine, because that intervention best mimicked the difference in plasma amino acid concentration between wild-type and F/A2 mice (14, 16).

The restriction of the effects of arginine deficiency to activation of the GCN2 stress-kinase pathway appears to reflect a limited increase in eIF2α phosphorylation, because maximizing the degree of phosphorylation of eIF2α with salubrinal caused a severe suppression of S6K1 phosphorylation and a disproportionate up-regulation of *Atf4*, *Chop*, *Gadd34*, and *Cat-1* expression. These data suggest that crosstalk of the stress-kinase and the mTORC1 pathways only occurs when eIF2α phosphorylation reaches an unphysiologically high level. *Gcn2* knockdown in C2C12 myoblasts almost completely prevented myotube formation, but did not affect the degree of phosphorylation of eIF2α, S6K1, or 4EBP1. Treatment with the growth factor IGF1 also did not affect eIF2α, S6K1, or 4EBP1 phosphorylation in response to arginine withdrawal. Our findings in C2C12 cells, therefore, support the hypothesis that arginine deficiency selectively affects the GCN2 stress-kinase pathway.

Arginine Deficiency Selectively Induces the Integrated Stress Response in Vivo—Based on the degree of phosphorylation of eIF2α and the expression of *Chop* mRNA, the GCN2 signaling pathway was activated in all F/A2 tissues tested on postnatal day 1. *Atf4* mRNA was only up-regulated in skeletal muscle of 1-day-old F/A2 mice, but ATF4 protein concentration was elevated in all tissues. These findings underline the severity of the muscle phenotype in F/A2 mice and the primarily translational regulation of ATF4 expression (52, 53). The concentration gradient of arginine between plasma and muscle was similar in control and F/A2 mice (4–5-fold), but the absolute concentrations were 3–4-fold lower in F/A2 than in wild-type mice, in agreement with an arginine supply rather than transport problem. F/A2 homozygotes resume growth around weaning (14), in all likelihood due to the increasing cellular arginine, IGF1, and IGF1R1 (64) concentrations in target tissues. In combination, these data, therefore, indicate that the GCN2 signaling pathway in muscle *in vivo* becomes activated when intracellular arginine concentrations in muscle decrease below ~400 μmol/kg. GCN2 activation in the neonatal intestine appears to have a similar

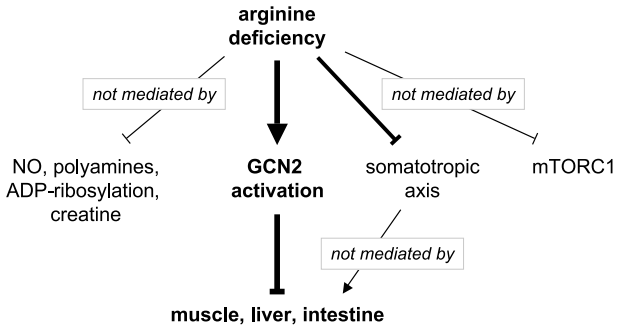

FIGURE 5. **Proposed mechanism underlying the arginine-deficient phenotype in mice.** Arginine is the precursor for NO, polyamines, and creatine synthesis, and regulates ADP-ribosylation, but these molecules do not mediate the developmental impairment of F/A2 mice. The somatotropic (GH/IGF1) axis does not function properly in arginine-deficient mice, but although GH supplementation increases *Igf1* mRNA concentration to wild-type values, it cannot restore growth. Arginine deficiency, finally, does not block mTORC1 phosphorylation-dependent signaling, but does activate the stress kinase GCN2 in neonatal and suckling F/A2 mice. The sole activation of the GCN2-mediated integrated stress response is apparently necessary and sufficient to block growth in a highly distinctive way.

sensitivity to arginine, but despite much lower tissue arginine concentrations, *Chop* mRNA and ATF4 protein concentrations in liver are similar to those in muscle and intestine.

Neonatal F/A2 mice did not show a reduced degree of S6K1 or 4EBP1 phosphorylation, which demonstrates that the effects of arginine deficiency are restricted to GCN2 activation both *in vitro* and *in vivo*. In this respect, arginine deficiency differs from leucine deficiency, which, at least in adult mice, causes both a GCN2-dependent phosphorrylation of eIF2α and a hypophosphorylation of S6K1 and 4EBP1 (65).

GCN2 Is Necessary to Cope with and Survive Neonatal Arginine Deficiency—The absence of an increase in eIF2α phosphorylation and *Chop* mRNA expression in neonates of crosses of F/A2 and *Gcn2*-deficient mice confirmed our *in vitro* data that GCN2 was necessary to mediate the response to arginine deficiency. These data also highlighted the vital role of GCN2 activation for postnatal survival of arginine-deficient pups. When a leucine-deficient diet was fed to pregnant $Gcn2^{+/-}$ dams, neonatal survival of their *Gcn2*-deficient offspring was only ~40% (22), but F/A2 neonates show a complete loss of vitality when the concentration of arginine falls to ~30% of normal. We have not yet identified the dysfunction that kills *Gcn2*-deficient F/A2 pups, but, in all likelihood, the decline in plasma glucose concentration, possibly in conjunction with an increase in plasma IGF1 concentration, is a contributing factor to their untimely death. A similar correlation between stress-kinase signaling and lethal hypoglycemia was also noted in pups carrying the phosphorylation-resistant Ser51Ala mutation of eIF2α (66), but these latter pups died earlier (<18 h) and with lower blood glucose concentrations ($\leq$1 mM) than *Gcn2*-deficient F/A2 neonates (<48 h and <3 mM, respectively). Apparently, GCN2-independent phosphorylation of eIF2α is also necessary to survive neonatally.

Arginine Is a Signaling Molecule in Its Own Right—The discussion of arginine-dependent signaling in tissues often focuses on arginine metabolites, such as NO, agmatine, polyamines,

[5] S. E. Kohler, unpublished observations.

creatine, or on arginine-mediated modifications such as ADP-ribosylation. This and earlier studies of the F/A2 mouse (14–17) demonstrate that the arginine-deficient phenotype is not mediated by a metabolite of arginine (Fig. 5). Instead, it is the low circulating concentration of arginine itself, which suppresses the somatotropic axis and activates the GCN2-signaling pathway without affecting mTORC1-mediated signaling. We would, therefore, like to propose that the striking phenotype of suckling F/A2 mice is a paradigm for selective GCN2 activation (Fig. 5).

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CHAPTER VII

GENERAL DISCUSSION

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In this thesis, I have described several genetically modified mouse models that allowed us to investigate arginine metabolism or to manipulate arginine availability. The models that were used affect arginine synthesis (conditional KO of argininosuccinate synthetase, *Ass*) or arginine degradation (conditional KO of arginase1, *Arg1*, and transgenic overexpression of rat arginase1 in mouse enterocytes, F/A2). Arginine metabolism in macrophages was reviewed (Chapter I) and an experimental analysis of mice with arginase 1 ablation in macrophages was described (Chapter III). The capacity of suckling mice to synthesize arginine in the enterocytes of the small intestine was confirmed for human infants (Chapter IV). Overexpression of arginase 1 in small intestinal enterocytes caused the surprisingly specific "neonatal arginine deficiency syndrome" (hypoargininemia, deficient muscle and hair growth and underdevelopment of Peyer's patches) in the suckling period (Chapter VI), whereas elimination of *Ass* in the same tissue did not (Chapter V). With the experience of generating several knockout models a troubleshooting guide was written (Chapter II).

Arginine is a substrate for the synthesis of proteins, ornithine, creatine, agmatine and nitric oxide (NO) (1). Arginine is also a secretagogue for growth hormone, insulin, glucagon and prolactin (2-4). Under normal conditions, endogenous arginine synthesis in adult mammals suffices to sustain daily requirements (5). A dietary source of arginine may, however, become necessary when demand increases under anabolic or catabolic conditions (5). For this reason, arginine is considered a conditionally essential amino acid.

Once inside cells, there are multiple pathways for arginine degradation to produce NO, ornithine, urea, polyamines, proline, glutamate, creatine and/or agmatine (1). These pathways are initiated by arginase, nitric oxide synthase, arginine:glycine amidinotransferase, and arginine decarboxylase. In mammals, the arginase pathway is quantitatively most important for arginine catabolism. Arginase 1 and 2 are encoded by two different genes, but hardly differ in their kinetic properties (6).

All arginine-metabolizing enzymes except arginase 2 are cytosolic and do potentially compete for their common substrate arginine (7, 8). The functionally most important competition is probably between arginase 1 and nitric oxide synthase (NOS). Therefore, relative changes in their enzymatic activities serve as major determinants of NO production in e.g. endothelial cells and macrophages (9). Both arginase and arginine decarboxylase activity can mediate the increased production of the polyamine precursors ornithine and agmatine, respectively. Agmatine is also an inhibitor of NOS activity. Though it is often claimed that arginase-dependent polyamine production is very important, *Arg1*-deficient macrophages (10) produced all polyamines more efficiently than wild-type macrophages (11). In line with our observations, *Arg1* and *Arg2* single and double KO mice did not differ from controls with respect to polyamine concentrations.

Arginine Deficiency

There are a number of arginine-deficiency syndromes, involving immune and endothelial dysfunction, depending on the disease context in which arginine deficiency occurs. For some disorders, elevated plasma arginase concentrations may be more important as a marker of organ damage than as a mechanistic component of the disease process. The liver contains the largest amount of arginase 1 in the body. Any disease or damage to the liver that results in elevated plasma levels of transaminases or other liver enzymes also will result in elevated plasma levels of arginase 1 (12-14). Enzymatically active arginase 1 is also present in red blood cells of humans (there is much less arginase in rodent red blood cells (15)), is released upon hemolysis, and remains active for some time following release into the plasma. For this reason, hemolytic blood samples contain low plasma arginine values (16). For the same reason, high concentrations of arginase and low concentrations of arginine are found in plasma samples of individuals with chronic hemolytic anemias, such as sickle cell disease (17-20) and paroxysmal nocturnal hemoglobinuria (20). Conflicting reports exist regarding plasma arginine concentrations in sepsis. Several studies suggest that plasma L-arginine concentrations are not decreased in sepsis (21, 22) or that they are more markedly decreased in trauma than in sepsis (23-26), but a recent meta-analysis concluded that plasma arginine concentrations are indeed reduced in sepsis in the absence of trauma or surgery (27).

Low values of the plasma [arginine]/([ornithine]+[citrulline]) ratio, the so-called “global arginine bioavailability ratio” (GABR), represent an independent risk factor for morbidity and mortality in sickle-cell and thalassemic patients (17, 28) and are also correlated with pulmonary hypertension in this patient population (17, 29). GABR values in asthma patients were, nevertheless, greater than in healthy controls, despite higher plasma arginase concentrations in the asthma group (30). Since plasma arginase activity was not determined, it is also possible that the arginase 1 protein in plasma may not have been active.

It has also been suggested, that cells expressing the cationic amino-acid transporter CAT1 can be depleted of their intracellular arginine if, there is a high ambient concentration of arginase (e.g.

due to cell lysis) or if ambient arginine is high in combination with a high intracellular arginase content in a neighboring cell (31), such as myeloid-derived suppressor cells (see Chapter I). This depletion results from concentration-dependent trans-stimulation of CAT1-mediated arginine transport by other cationic amino acids, such as ornithine or lysine. As a result, sensitive neighboring cells, such as T-cells, are rapidly depleted of arginine and become anergic (32, 33). The CAT1 transporter exhibits the highest trans-stimulation, but CAT2B and CAT3 also exhibit this property (34-36). As far as we know, however, none of these CAT isoforms is expressed.

Studies with cultured cells have demonstrated that arginine deficiency in the culture medium can result in T-cell dysfunction via reduced expression of the CD3ζ chain of the T-cell receptor complex (37) and cell cycle arrest (38), suggesting that similar effects might be observed in patients with arginine deficiency. Arginine deficiency would then result in an impaired immune response (39). Arginine deficiency also inhibits the maturation of B cells (40). Available evidence suggests that arginine is also required for defense against viruses, bacteria, fungi, malignant cells, and intracellular parasites (39). Although these findings suggest that arginine plays an important role in the immunity of (young) mice, our findings in a mouse model of severe arginine deficiency generated by arginase 1 overexpression in enterocytes demonstrates that arginine deficiency activates the general control non-derepressible 2 (GCN2) stress kinase pathway *in vivo* (41). Activation of the GCN2 kinase was recently also shown to underlie cell cycle arrest in T-cells (38). Indeed, the activation of the GCN2 kinase stress response pathway as a consequence of arginine deficiency has now been demonstrated in several cell types (38, 42). The T-cell dysfunction that is observed fin chronic inflammatory disease and after physical injury (e.g. trauma or surgery) is associated with increased arginase activity in human granulocytes (43-46) and may, therefore, also result from activation of the stress kinase pathway. The non-specific immune response in the presence of abundant arginine, therefore, appears to depend on NO production and that during arginine deficiency on the integrated stress response.

Benefits of arginine supplementation

Arginine is stable under sterilization conditions (e.g. high temperature and high pressure) and is not toxic to cells (47). Thus, its administration appears safe for animals and humans, although a recent study (48) reported a higher mortality of subjects receiving L-arginine. Many claims were raised to supplement arginine as a strategy to support arginine homeostasis and, thus, improve health and productivity under many physiological and pathological conditions. We cite a few examples with an emphasis on polyamines (reproduction), intestinal arginine synthesis (neonatal hyperammonemia), and NOS stabilization (endothelial dysfunction).

Polyamines, the polycationic products of arginine degradation, are thought to be important for cell growth and differentiation (see Chapter I for a more extensive discussion). Seminal fluid is abundant in polyamines (49). The oral administration of arginine-HCl (0.5g or 2.4 mmol/day) to infertile men for 6-8 weeks reportedly increased sperm counts and motility markedly in most patients and resulted in successful pregnancies (50). As usual, the underlying mechanism was not

studied, but was, in spite of the homeopathically low dose, suggested to be enhanced synthesis of polyamines and histones in sperm cells, and increased availability of NO for better sperm motility and capacitation (51). Similarly, arginine is claimed to extend the temporal window of oocytes for optimal fertilization (52) and to enhance embryonic and fetal survival and growth (53-58). As explanation, the stimulatory effects of polyamines and NO on angiogenesis and placental growth were mentioned (59). The beneficial effect of intravenous arginine supplementation on the reduction of spontaneous uterine contractility in women with the preterm onset of uterine contractions is explained by the relaxing effect of NO on smooth muscles (60).

Arginine deficiency in preterm infants (<30 μmol/L in plasma) is associated with severe hyperammonemia (61). This type of hyperammonemia often develops if the preterm infants are maintained on parenteral nutrition (62-64) and appears to predispose them to the respiratory distress syndrome (65) and necrotizing enterocolitis (66, 67). The reason for the observed arginine deficiency may well be that parenteral nutrition bypasses the gut. In newborn piglets, parenteral nutrition induces hypoargininemia, whereas infusion of the same solution into the gut prevents it, showing that (small-intestinal) enterocytes are a crucial source of *de novo* arginine synthesis (68, 69). Our data show the human small intestine acquires the potential to produce arginine well before fetuses become viable outside the uterus (70). The perinatal human intestine therefore resembles that of rodents and pigs in this respect. Supplementing arginine appears to prevent the hyperammonemia (61) and to counteract pulmonary hypertension (71) and necrotizing enterocolitis (72) in preterm infants.

NO deficiency is a major factor contributing to endothelial dysfunction, which occurs in a variety of metabolic disorders, including diabetes, hypercholesterolemia, hypertension, smoking and malaria (73). Dietary supplementation with arginine or watermelon (rich in citrulline) increased circulating levels of arginine, endothelial BH4 (co-factor of NOS) availability and NO synthesis, and enhanced endothelium-dependent relaxation in Zucker diabetic fatty rats (74). Hypertension in adult rats with intestinal resection (taking away the animal's capacity to produce citrulline (75) or patients with lysinuric protein intolerance (deficiency of the y^+L amino acid transporter (*Slc7a7* gene) which causes a rare autosomal recessive defect of dibasic amino acid transport resulting in a L-arginine deficiency (76)) could be prevented by parenteral administration of arginine. Either arginine or BH4 becomes deficient, NOS "uncouples" and releases free superoxide (O_2^-) radicals (77), which enhance oxidative stress and BH4 oxidation (78). This mechanism is responsible for many of the observed features and may explain why arginine supplementation may not only cause recovery of endothelial NO synthesis and reduction of superoxide production but also reduction of vascular oxidative damage and inhibition of platelet adherence and aggregation, leukocyte adherence to the endothelium, and inhibition of the proliferation of vascular smooth muscle cells (73, 79). The beneficial effects of arginine supplementation on renal diseases that cause systemic hypertension (80, 81), gastric ulcer healing (82), (hepatic) ischemia/reperfusion injury (83-85) and pulmonary function in patients with cystic fibrosis (86) probably all have a similar mechanistic basis.

In some cases, arginine supplementation appears to exert its beneficial effects via more than one mode of action. The beneficial effect of subcutaneous administration of arginine at the site of foot ulcers in diabetic patients (87) may involve both enhanced proline and polyamine synthesis, and NO-dependent increases in blood flow and nutrient supply. The basal arginine and ornithine fluxes are distinctly higher in burn patients than in healthy controls, conforming an accelerated turnover of arginine after burn injury (88). The function of arginine may also change with the progress of the condition it affects. The majority of *in vivo* studies showed that arginine supplementation from the time of tumor induction or inoculation was protective (89), whereas arginine supplementation during the promotion stage stimulated (colorectal) tumor growth (90).

Arginine or citrulline supplementation

If a dysfunction or disease is caused by deficiency of arginine, it should be ameliorated or reversed by supplementation with arginine or its precursor citrulline. As a practical consideration, the efficacy of enteral arginine supplementation is limited by gastrointestinal discomfort following ingestion of large amounts of arginine and by catabolism of a significant fraction of ingested arginine due to the huge arginase content of the body (91). The trans-stimulatory property of CAT transporters can also cause intracellular arginine deficiency if the extracellular concentration of ornithine or lysine is high (see earlier and Chapter I). Although dietary L-arginine supplementation enhanced the blood flow response to acetylcholine in some studies (92), it did not lead to significant improvements of endothelium-dependent vasodilation and blood flow in others (93). In most studies arginine supplementation increased plasma arginine concentrations, but functionally had no beneficial effects (94, 95). These disperse findings with respect to the functional benefits made arginine supplementation highly controversial (96-99).

For these reasons, citrulline has been advanced as an indirect source of arginine. Supplementation with citrulline efficiently increases the circulating and tissue arginine concentration, and mediates functional effects, such as NO production, without (thus far) side effects (95, 100, 101).

Arginase enzyme inhibitors or siRNAs that inhibit expression arginase or its transcription factors (102) represent a potential alternative for treatment of arginine deficiency, but side effects, such as inhibition of arginase function, require selective uptake in target cells and for that reason clearly limit its applicability.

Conclusion

Arginine deficiency is detrimental to many cell functions and tends to activate the powerful integrated stress-response pathway. The high sensitivity to catabolism by arginase and the peculiar characteristics of arginine transport across the cell membrane limit the applicability of arginine supplementation. Presently, citrulline supplementation appears to be a more promising

strategy. These findings show that intensive-care and pediatric physicians should have a basic, but up-to-date knowledge of arginine metabolism and transport.

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NEDERLANDSTALIGE SAMENVATTING

In dit proefschrift komen verschillende aspecten van het arginine metabolisme aan de orde. De rol van arginine en arginase in de synthese van ureum mag als bekend worden verondersteld. Echter, arginine en arginase spelen ook een zeer belangrijke rol in de fysiologie van macrofagen (***hoofdstuk 1***). Macrofagen zijn een heterogene groep cellen die ingedeeld worden op basis van hun weefselverdeling, activiteit, en oppervlaktemarkers. In navolging van de T_H1/T_H2 naamgeving worden ze vaak ingedeeld als klassiek (M1) of alternatief (M2) geactiveerde macrofagen. M1 macrofagen zouden vooral verantwoordelijk zijn voor het opruimen van tumorcellen en micro-organismen, terwijl M2 macrofagen betrokken zijn bij wondgenezing, doden van parasieten en immuun regulering. Omdat de weefseldistributie en het fenotype snel kunnen veranderen in reactie op omgevingsfactoren en vergelijkbare functies teweeggebracht kunnen worden door verschillende factoren, past een classificering op grond van functies beter.

De verschillende typen macrofagen verschillen in hun gebruik van arginine voor stikstofoxide (NO via NO synthases) en ornithine synthese (via arginase). NOS2 is de belangrijkste NOS isoform in macrofagen die tumorcellen en micro-organismen opruimen, terwijl arginase1 m.n. in macrofagen wordt aangetroffen die betrokken zijn bij wondgenezing, het opruimen van parasieten en de immuun regulering. In muizenmacrofagen wordt de expressie van arginase sterk gestimuleerd door de T_H2-type cytokines IL4, IL10, IL13 en TGFβ, terwijl arginase in humane macrofagen alleen door eencellige parasieten wordt geïnduceerd. Om de betekenis van arginine voor het functioneren van macrofagen beter te begrijpen hebben wij genetisch gemodificeerde muismodellen gemaakt waarin arginase tot overexpressie komt of waarin arginase of argininosuccinaat synthetase weefselspecifiek kan worden geïnactiveerd (***hoofdstukken 2,3,5, en 6***).

De ontwikkeling van technieken om de nucleotidenvolgorde van een gen in embryonale stamcellen te modificeren stelt ons in staat om de expressie van deze genen in levende organismen, meestal muizen, te veranderen. Vooral uitschakeling (knock-out) of vervanging (knock-in) van genen is een krachtig instrument gebleken. In ***hoofdstuk 2*** bespreken we daarom onze ervaringen met het maken van 3 zogenaamde conditionele knock-out muizen. Uitschakeling van de expressie van een gen in slechts één weefsel is nodig als deletie in alle cellen niet met het leven verenigbaar is. Daarnaast is een dergelijke muis beter geschikt om de functie van het betreffende gen in één type weefsel of op een bepaald moment in het leven te onderzoeken. Het maken van een conditionele knock-out muis vergt ten minste 2 jaar en bestaat uit een relatief groot aantal opeenvolgende stappen die elk voor zich “haken en ogen” hebben. Dit hoofdstuk beschrijft onze ervaring met de constructie van de vectoren welke nodig zijn voor het wijzigen van de nucleotidenvolgorde van een gen en de protocollen om het relatief geringe aantal embryonale stamcellen dat per “targeting” op de juiste wijze gewijzigd is op te sporen en te vermeerderen. Door de komst van de “CRISPR” (clustered regularly interspaced short palindromic repeats, een gen editerend systeem) en “TALENs” (transcription activator-like

effector nucleases, een soort restrictie-enzymen) is de opzet van targeting vectoren sterk veranderd. De daarop volgende selectie en vermeerdering van de juiste stamcel is nog grotendeels hetzelfde als toen dit overzicht werd samengesteld.

In ***hoofdstuk 3*** wordt de eerste toepassing van de door mij vervaardigde conditionele *arginase1* knock-out muis beschreven. Astma is een chronische ontsteking van de kleine luchtwegen, vaak als gevolg van overgevoeligheid voor een bepaald allergeen. De expressie van arginase1 in macrofagen is vrijwel afwezig in de long van controle muizen en zeer sterk verhoogd in de longmacrofagen van muizen waarin door sensibilisering tegen het kippeneiwit ovalbumine astma is opgewekt. Met behulp van een stabiele variant van acetylcholine kan de reactiviteit van de luchtwegen getest worden. In deze studie hebben we de expressie van arginase1 in macrofagen uitgeschakeld met zogenaamde Tie2Cre (uitschakeling in alle hematopoietische cellen) en LysMCre (uitschakeling in allen de macrofagen) transgenen. Helaas bleek LysMCre slechts in ~60% van de macrofagen voldoende actief om *arginase1* te elimineren, terwijl dat 96-98% was voor Tie2Cre. De studie rapporteert alleen de effecten in mannelijke muizen waarvan het *arginase1* gen met Tie2Cre is uitgeschakeld. Longfunctie werd met de "Flexivent" gemeten. Uitschakeling van *arginase1* in macrofagen veranderde de allergische reactie op ovalbumine sensibilisering en de weerstand in de grotere luchtwegen niet, maar verbeterde wel de functie van de kleinere luchtwegen. Voorts was de expressie van andere arginine-verbruikende enzymen en van arginine transporters, van de chemokines *Ccl2* en *Ccl11*, van de pro-inflammatoire cytokines *Tnfa* en *Ifng*, en van de epitheliale markers *Ccla3* en *Muc5ac* verminderd. De expressie van de voor astma typische genen *Il4*, *Il5*, en *Il13* was echter niet veranderd. Analyse van de correlaties tussen de verschillende parameters liet duidelijk zien dat arginase deficiëntie in macrofagen de coördinatie van de reactie op ovalbumine sensibilisering verminderde. De verwachte vermindering van de ontstekingsreactie werd dus niet waargenomen.

Moedermelk bevat relatief weinig arginine, maar aminozuren waaruit arginine gemaakt kan worden, zoals proline en glutamine, zijn rijkelijk aanwezig. De dunne darm van knaagdieren en varkens produceert arginine uit proline in de zoogperiode. Voorts lijden parenteraal gevoede zuigelingen vaak aan een te lage concentratie arginine (en daardoor een te hoge concentratie ammoniak) in hun bloed. Dit deed ons de vraag stellen of de dunne darm van de mens ook arginine zou kunnen maken. Om dit aannemelijk te maken beschrijven we in ***hoofdstuk 4*** metingen aan darmdoorsneden van 89 kinderen en volwassenen die semi-kwantitatief aangekleurd zijn op de aanwezigheid van de cruciale enzymen carbamoylfosfaat synthetase (CPS), ornithine aminotransferase (OAT), argininosuccinaat synthetase (ASS), arginase-1 en -2 (ARG1 en ARG2), en NO synthases (NOS1, -2, en -3). De CPS en ASS concentraties waren hoog tussen 23 weken zwangerschap en 3 jaar na de geboorte en daalden in de volgende 2 jaar tot het volwassen niveau. De OAT concentratie daalde ook, maar meer geleidelijk, terwijl ARG1 niet aantoonbaar was en ARG2 in de neonatale periode tot het volwassen niveau steeg. De

neuronen van de enterische plexus brachten ASS, OAT, NOS1 en ARG2 tot expressie, terwijl de zenuwen in de muscularis propria alleen ASS en NOS1 tot expressie brachten. Het endotheel van de arteriolen bracht ASS en NOS3 tot expressie en de gladde spierlaag eromheen OAT en ARG2. Op grond van deze bevindingen hebben we geconcludeerd dat de dunne darm van de mens ook de capaciteit heeft om arginine te maken. De expressie van ASS verdwijnt, net als die van lactase, geheel tussen 3 en 5 jaar na de geboorte, wat suggereert dat dat de normale speentijd van de mens als soort is.

Omdat alle tot nu toe bestudeerde zoogdieren, zoals in het vorige hoofdstuk is beschreven, in hun zuigelingenperiode alle enzymen die nodig zijn om arginine te maken in de enterocyten van de dunne darm tot expressie brengen, hebben we het enzym argininosuccinaat synthetase met behulp van het VillinCre transgen specifiek in deze enterocyten uitgeschakeld (***hoofdstuk 5***). De volledigheid van de uitschakeling in de enterocyten is vervolgens immunohistochemisch aangetoond (er wordt ook argininosuccinaat synthetase in de zenuwen van de darm tot expressie gebracht; zie vorige hoofdstuk). Onverwacht bleken deze muizen echter geen tekort aan arginine in hun bloed te hebben. We hebben daarom de flux van aminozuren in de darm en de lever bepaald. Hiervoor werd ook de arteriële bloedstroom naar de darm, de lever, en de nieren gemeten in 14-dagen oude muizen (respectievelijk 86%, 14% en 33% van de totale bloedstroom door de lever). Door de deletie van *Ass* in de enterocyten verdween de netto arginine synthese in de darm, terwijl de citrulline synthese verdubbelde. Voorts nam de arginine productie in de lever af of veranderde zelfs in een opname. Deze veranderingen in de lever vinden normaliter pas na het spenen plaats, als de darm niet langer arginine produceert. In overeenstemming met deze waarnemingen nam de expressie van enzymen die betrokken zijn bij de stofwisseling of het transport over de levercelmembraan toe, terwijl (behalve uitschakeling van *Ass*) geen veranderingen in gen expressie in de enterocyten werden gevonden. Deze waarnemingen wijzen er dus op dat de voortijdige (genetische) verwijdering van arginine biosynthese uit de enterocyten leidt tot een premature inductie van het patroon van aminozuurstofwisseling in de lever dat normaliter pas na het spenen wordt gezien.

In ***hoofdstuk 6*** is gezocht naar een mechanisme dat het fenotype van “F/A2” muizen verklaart. Deze transgene muizen brengen arginase1 tot overexpressie in de enterocyten van de dunne darm en hebben een fenotype dat lijkt enigermate lijkt op een deficiëntie van het groeihormoon IGF1 (insulin-like growth factor-1; te laag plasma arginine gehalte, verminderde haargroei, en onvolledige rijping van B-cellen). Lage arginine concentraties bleken de secretie van groeihormoon (GH) in hypofysecellen te remmen en de concentratie van Igf1 mRNA in de lever en het plasma IGF1 gehalte te verlagen, maar niet dat in de atrofische spieren. GH suppletie van de muizen deed weliswaar het Igf1 mRNA gehalte in de lever toenemen, maar niet de groei van de F/A2 muizen. Arginine depletie in het kweekmedium van de C2C12 spiercellijn activeerde voorts GCN2 signalering, maar liet dat van mTORC1 ongemoeid. GCN2 signalering, een

endoplasmatisch reticulum stress response, wordt geactiveerd door deficiëntie van belangrijke aminozuren. In homozygote F/A2 neonaten bedragen de plasma en weefsel concentraties van arginine slechts ~25% van de wild-type waarden. *Gcn2*-deficiente F/A2 muizen sterven kort na de geboorte door hypoglycemie, zodat activering van GCN2 kennelijk essentieel is voor het overleven van F/A2 neonaten. Omdat "downstream" effecten van andere stress kinases (eIF2α fosforylering, *Chop* expressie) niet verhoogd waren in *Gcn2*-deficiente F/A2 muizen, konden de effecten van arginine deficiëntie aan GCN2 signalering worden toegeschreven.

In ***hoofdstuk 7*** ten slotte worden pro's en con's van arginine en citrulline suppletie om een arginine deficiëntie te bestrijden besproken.

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